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CXLV. THE ACTION OF SUBSTANCES ALLIED TO 4:4′-DIAMINODIPHENYLSULPHONE IN STREPTOCOCCAL AND OTHER INFECTIONS IN MICE

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It was shown [Gray *et al.* 1937] that the therapeutic action of *p*-aminobenzenesulphonamide (sulphanilamide) persisted undiminished in certain of its derivatives, which were considerably less toxic to the host than the parent substance. It was found later [Buttle *et al.* 1937; Fourneau *et al.* 1937, 1] that 4:4′-diaminodiphenylsulphone was much more active than sulphanilamide, but also much more toxic, and the present paper describes attempts to reduce the toxicity relative to the activity in a similar way.

No substance of greater activity has been obtained during this investigation, but some of the derivatives described are almost equally active and much less toxic to mice.

A few more derivatives of sulphanilamide are included in the study.

For the sake of brevity the activity has been expressed as a fraction of that of 4:4′-diaminodiphenylsulphone, representing the dose of the latter which would produce a therapeutic effect in streptococcal infections of mice approximately equal to that of a unit quantity of the substance under test. Thus, if a substance is said to have 1/100 of the activity of the diaminodiphenylsulphone, 10 mg. of it will produce the same effect as 0·1 mg. of the latter. The technique used in testing the therapeutic effect of these substances has already been described [Buttle *et al.* 1936].

Effect of constitution on physiological action

A. *Derivatives of* 4:4′-*diaminodiphenylsulphone*

Group 1. *Modification of the sulphone group.* (*a*) *Diamino derivatives.* 4:4′-Diaminodiphenylsulphoxide [Smiles & Gazdar, 1908] has slightly less activity than the corresponding sulphone (between 1 and 1/10) [see also Fourneau *et al.* 1937, 2, and Levaditi & Vaisman, 1937] and the sulphide is still less active (between 1/10 and 1/100). The toxicity is not diminished in parallel with the activity; it is about the same for all these compounds; single doses of 5 mg. by mouth are tolerated by 20 g. mice but produce slight signs of poisoning; 10 mg. and 20 mg. doses kill some of the animals, and a 40 mg. dose is uniformly lethal. Acetylation of the amino-group of the sulphoxide does not reduce the activity appreciably as it does in the case of the sulphone. 4:4′-Diacetaminodiphenyldisulphone [Child & Smiles, 1926] has only a trace of activity, and is non-

[1] All animal experiments were performed by G. A. H. Buttle and D. Stephenson.

toxic in 100 mg. doses. 4:4′-Diaminodiphenyldisulphoxide [Bere & Smiles, 1924] is slightly less than 1/100 as active as the sulphone, and slightly greater in toxicity. 4-Aminophenyl-4′-aminobenzenesulphonate, in which the group $—SO_2.S—$ of the previous compound is replaced by $—SO_2.O—$, is quite inactive, as is also the acetyl derivative [Child & Smiles, 1926]. 4:4′-Diaminodiphenyldisulphide [Bere & Smiles, 1924] has less than 1/25 of the activity of the sulphone and is much more toxic. 4:4′-Diaminodiphenyloxide [Haeussermann & Teichmann, 1896] and 4:4′-diaminodiphenylmethane are completely inactive.

(*b*) *Dinitro-derivatives.* Whilst 4:4′-dinitrodiphenylsulphone has about 1/20 the activity and toxicity of the diamino-compound [Buttle *et al.* 1937; Fourneau *et al.* 1937, 1], the sulphoxide [Witte, 1932] has the same activity and toxicity as the corresponding sulphone, and the sulphide [Nietzki & Bothof, 1894] is only very slightly active. 4:4′-Dinitrodiphenylmethane [Staedel, 1894] and 4:4′-dinitrodiphenyloxide [Haeussermann & Teichmann, 1896] have a trace of activity.

4:4′-Dinitrodiphenyldisulphide [Willgerodt, 1885] has between 1/40 and 1/400 of the activity of the diaminodiphenylsulphone.

Group 2. *Substitution in the amino-group.* (*a*) *Acyl-substituents.* 4:4′-Diacetaminodiphenylsulphone [Fromm & Wittmann, 1908] has already been examined by Fourneau *et al.* [1937, 3] and by Bauer & Rosenthal [1938]. Our results are in general agreement with those of these authors. It is non-toxic to mice (800 mg. can be given by mouth to 20 g. mice) and has about ten times the activity of sulphanilamide in the streptococcal infection.

Table I shows that the lower doses of the diacetyl-compound approximate in activity to equivalent doses of the parent substance, but although the former can be given in large doses there is no increase in therapeutic effect with doses

Table I. *Comparison of sulphanilamide, 4:4′-diacetaminodiphenylsulphone and 4:4′-diaminodiphenylsulphone in haemolytic streptococcal infection of mice.*

	Daily dose of each drug in mg.						
	250	50	10	2	1·0	0·4	0·08
Sulphanilamide	—	22	11	3	—	—	—
4:4′-Diacetaminodiphenylsulphone	>24	>24	21	11	—	7	1
4:4′-Diaminodiphenylsulphone	—	—	—	—	>24	7	1

Table I. The figures in the table are the times in days elapsing before the death of three mice in each group of six.

Infection. All mice were infected intraperitoneally with 10^{-5} ml. of culture of haemolytic streptococcus (Richards).

Controls with 10^{-5} ml. of culture all died in 24 hr.; 5/6 controls with 10^{-9} ml. died in 3 days.

Treatment of the mice by mouth immediately after infection and doses repeated on the 1st, 2nd 3rd, 4th, 6th, 8th and 11th days.

above 10 mg. per day. This appears to be due to the slow rate of absorption or conversion into the active substance (possibly 4:4′-diaminodiphenylsulphone); any excess above 10 mg. is probably excreted unchanged. This diacetyl-compound does not give quite such good results as sulphanilamide when the treatment of the mice is delayed. In pneumococcal infections (Type I) we have only obtained a slight delay in the time of death of infected animals treated with the diacetyl-compound, while Fourneau *et al.* [1937, 3] obtained a considerable therapeutic effect.

When the diacetyl-compound is given to monkeys by mouth, only minute traces of diazotizable substance can be detected in the blood and no bactericidal effect is induced in this blood. On the other hand, sulphanilamide and 4:4′-diaminodiphenylsulphone both produce pronounced effects. It seems likely that

the active substance is eliminated at almost the same rate as it is formed; it is possible therefore that there may be a bactericidal effect in the tissue fluids whilst it is not detectable in the blood. The urine of these monkeys contains a diazotizable substance and will protect mice when given by mouth. Nitti (personal communication) obtained similar results.

In view of the lack of bactericidal effect in the blood, and of the fact that we do not get a better effect with this compound than with sulphanilamide in mouse infections when treatment is delayed, we doubt whether the 4:4′-diacetaminophenylsulphone would be superior to sulphanilamide in clinical treatment of severe infections, e.g. streptococcal septicaemia. On the other hand, where a prolonged effect is required in more chronic conditions, it may possibly prove to be a help to clinicians owing to its lower toxicity, together with the lower dose which is therapeutically effective.

The dimyristyl- and dilauryl-derivatives of the diaminosulphone are inactive. The di-(aminobenzenesulphonyl)-derivative has 1/100 of the activity of the parent substance, i.e. it has the same activity as sulphanilamide. Thus it appears that it is disadvantageous to bring these therapeutically active groups together in one molecule. 4:4′-Disulphonamidodiphenylsulphone is completely inactive.

(*b*) *Schiff's bases.* We have prepared a number of anils in which one or both of the amino-groups of the diaminosulphone are condensed with aromatic aldehydes. Of these the benzylidene-compound is the most interesting. It is more toxic than the diacetyl-compound (single doses of 50 mg. are tolerated, but 200 mg. are lethal to 20 g. mice; repeated daily doses of 10 mg. can be given to infected mice), is about half as active as the parent substance in streptococcal infections of mice, and induces a bactericidal effect against streptococci in the blood of the monkey when given by mouth.

This compound produces a better therapeutic effect in pneumococcal infections type I [Buttle, 1937] and type II of mice than any other compound we have used. The effect on type III pneumococcal infection in mice is also better than that with sulphanilamide.

The benzylidene-derivative in 10 mg. doses has a better effect in pneumococcal infections than 1 mg. doses of the sulphone. This is the only instance we know where the larger dose of a less toxic derivative of this series has a better effect than the maximal tolerated dose of the parent substance.

The anisylidene-, piperonylidene-, dicinnamylidene- and dimethylaminobenzylidene-derivatives have all about the same activity in streptococcal infections of mice as the foregoing compound; the toxicity is similar or slightly less in the cases of the anisylidene- and dicinnamylidene-, and definitely less in the cases of the piperonylidene- and dimethylaminobenzylidene-derivatives (200 mg. doses of the last are tolerated). These compounds, like the *N*-diacetyl-derivative mentioned above, do not induce a bactericidal effect in the blood of the monkey.

Di-*o*-carboxybenzylideneaminodiphenylsulphone has only about 1/100 of the activity of the parent substance in streptococcal infection; it is thus much less active than any of the other Schiff's bases.

The sodium bisulphite compound of 4:4′-dicinnamylideneaminodiphenylsulphone is freely soluble in water and has about 1/20 of the therapeutic activity of the parent substance when given by mouth; when given subcutaneously it is slightly more effective than an equal quantity of the corresponding derivative of sulphanilamide, but it is also slightly more toxic.

A very soluble substance, the constitution of which is still under investigation, is obtained by heating 4:4′-diaminodiphenylsulphone with an aqueous solution

of glucose. When given by mouth this has about half the activity of the parent substance; 50 mg. are tolerated by 20 g. mice and 100 mg. are lethal. When given subcutaneously the substance is less active and also less toxic than when it is given by mouth, daily doses of 150 mg. being well tolerated. It seems likely that in the latter case it is more rapidly converted into the parent sulphone by the gastric juice. The glucose derivative is more active as a therapeutic agent for subcutaneous administration than any of the other soluble derivatives of the sulphone or of sulphanilamide which we have tried so far. Like the benzylidene Schiff's base, it produces a good therapeutic effect in pneumococcal infections of mice, and in most of our experiments has proved only slightly inferior to the latter.

(*c*) *Aralkyl derivatives.* Tetrabenzyldiaminodiphenylsulphone is inactive.

Group 3. Additional substituents, e.g. nitro-, chloro-, amino-groups. All these substituents have a dystherapeutic effect. The insertion of nitro-groups in positions 3 and 3′ in the diaminosulphone [Ullmann & Korselt, 1907] produces a substance which has only a trace of activity. 3:3′-Diamino-4:4′-dihydroxydiphenylsulphone [Annaheim, 1874, 1875] is completely inactive.

The effect of the presence of nitro-groups in positions 2 and 2′ in 4:4′-dinitrodiphenylsulphone and the corresponding sulphide [Beilstein & Kurbatow, 1879], is to reduce the activity to a trace in the first case and to destroy it in the other. 2:4:6-Trinitro-4′-acetaminodiphenylsulphone has only a trace of activity.

Group 4. Substituents other than amino-groups. 4:4′-Dichlorodiphenylsulphone [Ullmann & Korselt, 1907], 4:4′-dihydroxydiphenylsulphone [Glutz, 1868; Annaheim, 1876], and 4:4′-dihydroxy-3-3′-dinitrodiphenylsulphone [Glutz, 1868], are completely inactive.

Group 5. Unsymmetrical compounds. 4-Nitro-4′-aminodiphenylsulphone [see also Fourneau *et al.* 1937, 2] has slightly less activity than the diamino-compound and the same toxicity. 4-Aminobenzylphenylsulphone [Waldron & Reid, 1923] is inactive while 4-aminophenylbenzylsulphone [Waldron & Reid, 1923] has a slight trace of activity. In each case the nitro- and amino-derivatives have equal activities. The presence of a further amino- or nitro-group in the *p*-position in the above amino- or nitro-compounds respectively [Waldron & Reid, 1923] does not influence the activity, except in the case of 4-nitrobenzyl-4′-nitrophenylsulphone, which is inactive.

(*a*) The series of compounds of the general formula: $R_1.C_6H_4.SO_2R_2$ ($R_1 = NH_2$ or NO_2), prepared by Waldron & Reid [1923], in which R_2 is an aliphatic chain of 1–4 carbon atoms, have been tested: the amino-compounds containing 2 or 3 carbon atoms in the chain have a trace of activity (between 1/100 and 1/1000 of that of the diaminosulphone); whereas those with 1 or 4 carbon atoms are inactive, as are all the corresponding nitro-compounds. These substances have also been tested by Fourneau *et al.* [1938] who have obtained similar results. It is interesting to note that the ethyl- and propyl-sulphones have the same activity as 4-aminodiphenylsulphone. On the other hand, 4-nitrodiphenylsulphone is as active as the corresponding amino-compound, whereas the aliphatic nitro-compounds are inactive.

(*b*) *Sulphoxides.* 4-Aminodiphenylsulphoxide can be resolved into optically active forms. We have prepared and tested the *d*-isomeride and also a mixture consisting of 47 % of *l*-isomeride with 53 % of the racemic form. The latter mixture was found to be more active than the *d*-isomeride, the ratio of activities is, however, less than 10 to 1; thus it appears that the *l*- is more active than the *d*-form.

B. *Derivatives of sulphanilamide, and miscellaneous substances*

The compound 4-aminobenzenesulphonamidophenyl-4′-sulphondimethylamide has been prepared. This compound has already been reported upon by Domagk [1937] and has been given the trade name "Uliron". Confirming the observations of Domagk we find that it is as active as sulphanilamide in the treatment of streptococcal infections in mice, while less toxic (a single dose of 800 mg. by mouth is tolerated by 20 g. mice, whereas mice will only withstand 50 mg. of sulphanilamide). In the treatment of staphylococcal infections, on the other hand, we find that the compound is not as effective as sulphanilamide.

Table II. *Comparison of sulphanilamide and 4-aminobenzenesulphonamidophenyl-4′-sulphondimethylamide ("Uliron") in staphylococcal infections*

Treatment	Culture ml. injected intraperitoneally with 5% mucin	Time for 60% deaths in groups of 5 mice (days)
Sulphonamide, 25 mg.	10^{-7}	>12
	10^{-5}	>12
	10^{-3}	7
"Uliron", 25 mg.	10^{-7}	>12
	10^{-5}	9
	10^{-3}	1
Controls with	10^{-7}	All died within 1 day
Controls with	10^{-9}	4/5 died in 5 days

Treatment by mouth twice daily for 6 days and once daily up to 14 days. The mice were observed for 12 days only after infection.

In this latter respect our findings do not agree with those of Domagk, who reports that it is more effective than sulphanilamide in staphylococcal infections, although no comparative data are given; on the other hand, Mellon *et al.* [1937] find that it is ineffective both experimentally and clinically.

Our results differ from those of Domagk in that we have only been able to protect mice against an infection by a very small number of staphylococci suspended in 5% mucin injected intraperitoneally, e.g. 10^{-5} ml. of culture containing about 50,000 cocci, whereas in Domagk's experiment a 1/2 dilution of culture was used.

In staphylococcal infections we have not found any compound which is more effective than sulphanilamide, although the benzylidene-derivative of the diaminodiphenylsulphone is almost equally so. Sulphanilamide is not nearly as good in the treatment of staphylococcal infections as it is in the treatment of streptococcal infections [Buttle, 1937]. In the latter case mice injected with 10,000 lethal doses of culture can be completely cured, whereas in staphylococcal infections most of the animals infected with 100 lethal doses recover and death is delayed for 2 weeks in those infected with 10,000 lethal doses. Clinical results [Colebrook & Purdie, 1937] show that sulphanilamide may have some effect in staphylococcal septicaemia. 2-Carboxybenzylideneaminobenzene-4-sulphonamide is equal to sulphanilamide in activity against the streptococcus and is less toxic (100 mg. by mouth are well tolerated). 4:4′-Diacetaminobenzenesulphonimide is almost inactive. 4-Sulphonamidophenylguanidine is equal to sulphanilamide in activity and toxicity.

Miscellaneous compounds

(*a*) *Sulphinic acids.* We find that *p*-acetaminobenzenesulphinic acid is active, whereas the corresponding sulphonic acid is not. 2-Pyrrolidone-5-carboxyaminobenzene-4′-sulphinic acid has the same activity as sulphanilamide and is

less toxic (100 mg. do not produce symptoms). While this paper was in course of preparation Gley [1937] described the activity of *p*-acetaminobenzenesulphinic acid.

(*b*) *Diphenyl derivatives.* Benzidine-*o*-sulphone [Griess & Duisberg, 1889] is inactive.

(*c*) Trithioaniline, sulphamide and aminosulphonic acid are inactive.

The following compounds were found to be inactive or to have only a slight trace of activity (in the latter case the approximate ratio of the activity to that of 4:4′-diaminodiphenylsulphone is stated in brackets): 2:5-dihydroxy-4′-acetaminodiphenylsulphone, 2:5-dihydroxy-4′-aminodiphenylsulphone (<1/1000), 4-nitrophenylmercaptan [Kehrmann & Bauer, 1896], benzyl-4-acetaminophenylsulphone, diphenylamine-*o*-sulphone [Bernthsen, 1906] (1/1000), di-(1-phenyl-2:3-dimethyl-5-pyrazolonyl)-4:4′-disulphide [Brand & Bausch, 1934] (1/500), 2:2′-dinitro-4:4′-diaminodiphenylmethane [Gram, 1892] (1/5000), tetramethyldiaminobenzophenone, 4:4′-diaminodiphenylcarbamide, 4-aminobenzenesulphonethanolamide [Kharasch & Reinmuth, 1937], 4-aminobenzenesulphonylglycine [Bauer & Rosenthal, 1938].

Of the substances mentioned above, the following appear to offer advantages for clinical use: 4-benzylideneamino-4′-aminodiphenylsulphone in pneumococcal infections, 4:4′-diacetaminodiphenylsulphone in cases where low toxicity is of primary importance, and the water-soluble glucose derivative for parenteral injection in cases when treatment by the mouth is impossible.

In considering the possible clinical value of derivatives of 4:4′-diaminodiphenylsulphone it must be pointed out that, although the substance is a hundred times as effective as sulphanilamide in the treatment of mouse streptococcal infections, it will not necessarily be of the same relative efficacy in the treatment of human infections. Sulphanilamide is excreted twice as rapidly in the mouse as in the rabbit, monkey or man; on the other hand, the sulphone is eliminated comparatively slowly in all the animals and consequently exercises a more persistent therapeutic action. The marked difference between the rate of elimination of sulphanilamide and of the sulphone is therefore not so obvious in the monkey and rabbit as it is in the mouse. In fact, in rabbits infected with streptococci the sulphone is only 10 times as active as the sulphonamide, a difference which has also been established when the two compounds are added to mixtures of human or monkey blood and streptococci *in vitro* [Buttle *et al.* 1937]. Thus it seems probable that the ratio of activities of the two compounds in human infections would be of the order of 10 to 1 as it is in the rabbit, not 100 to 1 as it is in the mouse.

H. C. Brown and J. C. Broom (personal communication) report that the 4:4′-diaminodiphenylsulphone-glucose derivative is inactive in *Leptospira icterohaemorrhagiae* infection of guinea-pigs or *Trypanosoma brucei* infection of mice.

Chemical section

Schiff's bases of 4:4′-diaminodiphenylsulphone

4-Benzylideneamino-4′-aminodiphenylsulphone. 4:4′-Diaminodiphenylsulphone (2 g.) dissolved in hot alcohol (10 ml.) was treated with benzaldehyde (1·64 ml. = 2 mol.), and the solution boiled. The anil soon commenced to separate in the pure state, in rosettes of needles, M.P. 232° (corr.) yield 2·42 g. = 89% of the calculated amount. It is very sparingly soluble in water and ordinary organic solvents, with exception of hot glacial acetic acid, recrystallization from which is attended by some decomposition. (Found: C, 68·2; H, 4·9; N, 8·4; S, 9·3%. $C_{19}H_{16}O_2N_2S$ requires C, 67·8; H, 4·8; N, 8·3; S, 9·5%.)

An attempt to obtain the corresponding monobenzyl derivative by hydrogenation in presence of platinum oxide of a suspension in acetic acid gave a product from which the pure substance could not be isolated.

4:4′-Tetra-N-benzyldiaminodiphenylsulphone. 4:4′-Diaminodiphenylsulphone (2·2 g.) was dissolved by heating in benzyl chloride (6·1 ml. = 6 mol.) and the solution boiled for 1 hr., for the first half alone, and then with addition of aqueous Na_2CO_3 (50 ml. of 35%). The oily layer was dissolved in acetone and dried over Na_2SO_4. On evaporation crystals separated; M.P. after recrystallization from acetone-alcohol, 200° (corr.). It is insoluble in water or alcohol, moderately soluble in acetone. (Found: C, 78·9; H, 6·2; N, 4·7%. $C_{40}H_{36}O_2N_2S$ requires C, 78·9; H, 6·0; N, 4·6%.)

4-p-Methoxybenzylideneamino-4′-aminodiphenylsulphone. 4:4′-Diaminodiphenylsulphone (2·48 g.), alcohol (15 ml.) and anisaldehyde (2·4 ml.), gave, by the procedure described for the benzylidene derivative, pale yellow needles, M.P. 228° (corr.) unchanged by crystallization from alcohol; yield 3·58 g. (98%). It is very sparingly soluble in alcohol. (Found: C, 65·8; H, 5·2; N, 7·9; S, 8·7%. $C_{20}H_{18}O_3N_2S$ requires C, 65·5; H, 5·0; N, 7·7; S, 8·8%.)

4-(3:4-Methylenedioxybenzylidene)-amino-4′-aminodiphenylsulphone. 4:4′-Diaminodiphenylsulphone (2 g.), alcohol (10 ml.) and piperonal (2·42 g.) were heated together with continual agitation for 75 min. The two liquid layers formed had then become miscible, and the anil began to crystallize out in needles, M.P. 232° (corr.), yield 3·04 g. (98%). It is practically insoluble in the ordinary organic solvents with the exception of glacial acetic acid. (Found: C, 63·5; H, 4·4; N, 7·5; S, 8·3%. $C_{20}H_{16}O_4N_2S$ requires C, 63·1; H, 4·2; N, 7·4; S, 8·4%.)

4:4′-Di-o-carboxybenzylideneaminodiphenylsulphone. *o*-Phthalaldehydic acid (2·7 g.) dissolved in boiling alcohol (15 ml.) was treated in one operation with finely powdered 4:4′-diaminodiphenylsulphone (2·23 g. = 0·5 mol.). The solution set to a paste; 15 ml. more of alcohol were added and boiling continued for 15 min. On cooling and filtering the pure substance was obtained in stout columns, M.P. 287° (corr.), yield 4·1 g. (89%). It is insoluble in water or the ordinary solvents, and cannot be dissolved in aqueous NaOH to a neutral solution. (Found: C, 65·4; H, 4·2; N, 6·0; S, 6·6%. $C_{28}H_{20}O_6N_2S$ requires C, 65·6; H, 3·1; N, 5·5; S, 6·3%.)

4:4′-Dicinnamylideneaminodiphenylsulphone. Cinnamic aldehyde (4 ml.; excess over 2 mol.) was quickly added with stirring to 4:4′-diaminodiphenylsulphone (1·24 g.) in the cold. The solid dissolved, and within a few minutes the solution set to a paste, which was ground under ether, dried, dissolved in the minimum amount of chloroform at room temperature and treated with an equal volume of acetone, when the anil separated in shining leaflets, M.P. 252° (corr.) yield 2·17 g. = 91%. It is very sparingly soluble in water or the ordinary solvents. (Found: C, 75·2; H, 5·1; N, 5·9; S, 6·8%. $C_{30}H_{24}O_2N_2S$ requires C, 75·6; H, 5·1; N, 5·9; S, 6·7%.)

Tetrasodium 4:4′-di-γ-phenylpropylaminodiphenylsulphone α:γ:α′:γ′-tetrasulphonate. 1·59 g. of the dicinnamylidene derivative, finely powdered, were digested at 100° with a solution of $NaHSO_3$ (1·39 g.) in water (7 ml.) for 90 min.; a small amount of flocculent material was removed and the solution evaporated. The syrup obtained was kept under alcohol for a day, then ground and washed with fresh alcohol and dried in a desiccator; yield 2·57 g. (86%). It is readily soluble in cold water giving a neutral solution. (Found: N, 3·1; S, 18·0%. $C_{30}H_{28}O_{14}N_2S_5Na_4$ requires N, 3·1; S, 18·0%.)

4-p-Dimethylaminobenzylidene-4′-aminodiphenylsulphone. *p*-Dimethylaminobenzaldehyde (2·4 g. = 2 mol.) and 4:4′-diaminodiphenylsulphone (2·0 g.) were finely ground together and heated for 4 hr. at 140°. The melt was powdered and extracted with boiling alcohol until a sample melted at 252° (corr.) when the product consisted of the pure monoanil, yield 1·05 g. (34%). It is scarcely soluble in water or the ordinary solvents, with the exception of amyl alcohol. (Found: C, 66·7; H, 5·7; N, 11·1; S, 8·5%. $C_{21}H_{21}O_2N_3S$ requires C, 66·5; H, 5·6; N, 11·1; S, 8·5%.)

N-*Acyl derivatives of 4:4′-diaminodiphenylsulphone*

4:4′-Dilaurylaminodiphenylsulphone. Lauric acid (25 g.) was refluxed 1 hr. with thionyl chloride (25 ml.). Excess thionyl chloride was then evaporated on the water bath *in vacuo* and the residual chloride dissolved in pyridine (50 ml.), when a crystalline compound separated immediately. This dissolved on the addition of a solution of 4:4′-diaminodiphenylsulphone (15 g.) in pyridine (50 ml.); the mixture was heated for 1 hr. on the water bath and then poured into water (1000 ml.). The amorphous precipitate was dissolved in chloroform (300 ml.) and washed succes-

sively with dilute NaOH, water, dilute HCl and finally with water; the dried chloroform solution evaporated to dryness and the residue crystallized from alcohol three times. The product then melted at 148° (uncorr). (Found: C, 71·0; H, 9·3; N, 4·6; S, 5·3%. $C_{36}H_{56}O_4N_2S$ requires C, 70·6; H, 9·2; N, 4·6; S, 5·2%.)

4:4′-Dimyristylaminodiphenylsulphone. This was made by a method similar to that used for the dilauryl-compound, omitting the washing with alkali and acid. The product was recrystallized to constant M.P. from alcohol, M.P. 140–141° (uncorr.). (Found: C, 72·0; H, 9·6; N, 4·4; S, 4·9%. $C_{40}H_{64}O_4N_2S$ requires C, 72·0; H, 9·6; N, 4·2; S, 4·8%.)

4:4′-Di-(p-*aminobenzenesulphonylamino*)-*diphenylsulphone.* 4:4′-Diaminodiphenylsulphone (9·8 g.), 4-acetaminobenzenesulphonyl chloride (10 g.) and anhydrous Na_2CO_3 (7 g.) were refluxed with chloroform (100 ml.) for 4 hr. The solid material was filtered off and thoroughly washed with water. After recrystallization from dilute pyridine this melted at 285° unchanged by further crystallization (Found: acetyl, 11·4%. $C_{28}H_{20}O_8N_4S_3$ requires acetyl, 13·6%.) The substance was probably contaminated with some of the monoacetaminobenzenesulphonyl-compound, as this had been obtained under similar conditions. The acetyl-compound was hydrolysed by boiling with 20% HCl for 20 min. after solution had taken place. On adding excess of ammonia to the solution the precipitate first formed dissolved almost completely. The small amount of insoluble material was found to be 4:4′-diaminodiphenylsulphone. The solution, neutralized with acetic acid, yielded the impure base as a white, amorphous powder. The hope that the base might be easier to purify than its acetyl-derivative was not realized as it could not be recrystallized. It was therefore reprecipitated, but as the product had very little bactericidal action it was not investigated further. (Found: N, 10·2; S, 16·0%. $C_{21}H_{22}O_6N_4S_3$ requires N, 10·0; S, 17·2%.)

Derivatives of 4-aminodiphenylsulphone

4-Nitro-4′-aminodiphenylsulphone. 4-Nitro-4′-acetaminodiphenylsulphide (4·8 g.) was dissolved in hot acetic acid (100 ml.) and $KMnO_4$ (2·5 g.) in hot water (15 ml.) added during 10 min. The dark brown solution was heated for 20 min. on the water bath, decolored with sulphurous acid and diluted with water. The product was filtered, washed and hydrolysed by boiling with water (30 ml.) and strong HCl (30 ml.) for 30 min., the solution diluted and made alkaline with ammonia, when the product separated as a yellow crystalline precipitate. After five recrystallizations from alcohol the M.P. was constant at 171° (uncorr.). (Found: N, 10·3; S, 11·9%. $C_{12}H_{10}O_4N_2S$ requires N, 10·1; S, 11·5%.) This substance was mentioned by Fourneau *et al.* [1937, 2] but not described.

Benzyl-4-acetaminophenylsulphone. To a solution of sodium *p*-acetaminobenzenesulphonate (2·2 g.) in water (3 ml.) were added, with stirring, benzyl chloride (1·15 ml. = 1 mol.) and alcohol (1 ml.), and the mixture was shaken for several hours. On standing it deposited 0·56 g. of solid, which was crystallized from alcohol, M.P. 185° (corr.). (Found: C, 61·9; H, 5·2; N, 5·0; S, 10·9%. $C_{15}H_{15}O_3NS$ requires C, 62·2; H, 5·2; N, 4·8; S, 11·1%.)

2:4:6-Trinitro-4′-acetaminodiphenylsulphone. *p*-Acetaminobenzenesulphinic acid (2 g.), picryl chloride (2·48 g.) and alcohol (10 ml.) were heated together on the water bath for a short time. The solid, which separated was washed well with alcohol and recrystallized from glacial acetic acid, needles, M.P. 268° (corr.), insoluble in water, alcohol and benzene, sparingly soluble in hot acetic acid. (Found: C, 41·4; H, 2·6; N, 13·5; S, 7·6%. $C_{14}H_{10}O_9N_4S$ requires C, 41·0; H, 2·5; N, 13·7; S, 7·8%.)

2:5-Dihydroxy-4′-acetaminodiphenylsulphone. *p*-Acetaminobenzenesulphinic acid (2 g.) was dissolved in water (25 ml.) on the water bath, and freshly distilled *p*-benzoquinone (1·08 g. = 1 mol.) gradually added. The suspension was heated for 1 hr., the solid dried and recrystallized from methyl alcohol, M.P. 282° (corr.). It is sparingly soluble in alcohol, acetone or acetic acid, insoluble in benzene or chloroform. (Found: C, 54·9; H, 4·5; N, 4·7; S, 10·7%. $C_{14}H_{13}O_5NS$ requires C, 54·7; H, 4·3; N, 4·6; S, 10·4%.)

2:5-Dihydroxy-4′-aminodiphenylsulphone. 2·75 g. of the acetyl-derivative were boiled for 1 hr. with 17% HCl (11·6 ml.). The dark solution was treated with charcoal and on standing deposited stout prisms, M.P. 232° (corr.), a solution of which in hot dilute HCl was quickly cooled and made alkaline with Na_2CO_3, when it deposited colourless needles, M.P. 180°. (Found: C, 54·6; H, 4·2; N, 5·5; S, 12·1%. $C_{12}H_{11}O_4NS$ requires C, 54·3; H, 4·2; N, 5·3; S, 12·1%.)

d-*4-Aminodiphenylsulphoxide.* It was shown by Harrison *et al.* [1926] that mixed sulphoxides should be capable of resolution into optical isomerides and they succeeded in resolving a number of

such sulphoxides, but 4-aminodiphenylsulphoxide was not included amongst these. *r*-4-Aminodiphenylsulphoxide (10·0 g.) [Hinsberg, 1903] was dissolved in a solution of *d*-camphorsulphonic acid (12·75 g.) in water (50 ml.) and the mixture concentrated to 30 ml. After some weeks crystallization took place and the product was recrystallized twice from three parts of 15% aqueous camphorsulphonic acid. Excess of acid is necessary to prevent precipitation of the base. The M.P. was then constant at 185° (uncorr.). By precipitation with ammonia this salt yielded a base, M.P. 171° (uncorr.) unchanged on recrystallization from alcohol. (Found: N, 6·4; S, 14·6; $[\alpha]_{5461}^{17°} = +157°$; $[\alpha]_{D}^{17°} = +127°$ (0·8% solution in alcohol). $C_{12}H_{11}ONS$ requires N, 6·5; S, 14·7%.)

In agreement with the general results of Harrison *et al.* it was found that the *d*-base had a much higher M.P. (171°) than the *r*-base (151°). The *l*-base was not isolated as the purpose of the resolution was to see if any difference in bactericidal action existed between the isomerides, and the *r*- and *d*-isomerides would suffice for this purpose if the difference were marked.

Sulphonamides and related substances

4-o-Carboxybenzylideneaminobenzenesulphonamide. A solution of *o*-phthalaldehydic acid (8·9 g.) and *p*-aminobenzenesulphonamide (10·6 g. = 1 mol.) in alcohol (50 ml.) was boiled for 6 hr. The pure anil gradually separated, M.P. 276° (corr.), yield 9·35 g. (49·7%). It is insoluble in solvents and cannot be dissolved in aqueous NaOH to a neutral solution. (Found: C, 55·4; H, 4·2; N, 9·2; S, 10·6%. $C_{14}H_{12}O_4N_2S$ requires C, 55·2; H, 4·0; N, 9·2; S, 10·6%.)

4-Sulphonamidophenylguanidine. *p*-Aminobenzenesulphonamide (20 g.) and dicyandiamide (8 g.) were heated together at 210° for 1·5 hr., the melt powdered and dissolved in dilute HCl, and the filtered solution made alkaline with ammonia. A sticky mass came down and the mother liquors when separated from this crystallized on standing. This crystalline material was recrystallized several times from boiling water and then had M.P. 185° (uncorr.). (Found: C, 39·4; H, 4·7; N, 26·0; S, 15·0%. $C_7H_{10}O_2N_4S$ requires C, 39·3; H, 4·7; N, 26·2; S, 15·0%.)

4:4′-Bis-(acetaminobenzenesulphon)-imide. Equal parts of *p*-acetaminobenzenesulphonylchloride and sodium *p*-acetaminobenzenesulphonamide were refluxed in 10 parts of benzene for 6 hr. The solid product was filtered off and ground with dilute Na_2CO_3. After filtration from insoluble material the solution was acidified and the required product obtained as a white precipitate, M.P. 270° (uncorr.). (Found: N, 10·2; S, 15·6; acetyl, 19·2%. $C_{16}H_{17}O_6N_3S_2$ requires N, 10·2; S, 15·6; acetyl, 20·9%.)

4:4′-Disulphonamidodiphenylsulphone. Diphenylsulphone-4:4′-disulphonylchloride was described by Bourgeois & Petermann [1903]. On stirring with strong aqueous ammonia it warmed up somewhat and gave the required amide. After two recrystallizations from 50% alcohol the M.P. was 288°, not changed by two further recrystallizations from alcohol. (Found: C, 38·8; H, 3·3; N, 7·6; S, 25·7%. $C_{12}H_{12}O_6N_2S_3$ requires C, 38·3; H, 3·2; N, 7·4; S, 25·6%.)

2-Pyrrolidone-5-carboxy-4′-aminobenzenesulphinic acid. Chlorosulphonic acid (16 ml.), cooled in running water, was treated gradually with *l*-2-pyrrolidone-5-carboxyanilide [Gray, 1928], the mixture heated at 60–70° for 3 hr., cooled and poured on ice. The resulting solid was treated with a solution of Na_2SO_3 (28 g.) in water (56 ml.) and the solution, after standing overnight, acidified with 60% H_2SO_4. The solid was well washed with water, M.P. 228°, unchanged by crystallization from water. It is insoluble in alcohol. (Found: C, 49·3; H, 4·5; N, 10·4; S, 11·9%. $C_{11}H_{12}O_4N_2S$ requires C, 49·2; H, 4·5; N, 10·5; S, 12·0%.)

4-Aminophenyl-4′-aminobenzenesulphonate. 4-Acetaminophenyl-4′-acetaminobenzenesulphonate (50 g.) [Child & Smiles, 1926] was mixed to a paste with strong HCl (200 ml.) and heated on the water bath for 30 min. On standing the hydrochloride of the base crystallized. The base was precipitated from the aqueous solution of the hydrochloride with ammonia, and after two recrystallizations from alcohol obtained as a white crystalline powder, M.P. 146° (uncorr.). Found: N, 10·7; S, 12·2%. $C_{12}H_{12}O_3N_2S$ requires N, 10·6; S, 12·1%.)

Summary

In an endeavour to find a drug similar in activity to 4:4′-diaminodiphenylsulphone but less toxic, a systematic investigation of a number of allied compounds has been carried out and certain relations established between structure and

pharmacological action. A number of Schiff's bases and acyl derivatives of this substance are described, as well as derivatives of 4-aminodiphenylsulphone and some substituted sulphonamides. None of these proved to be more active than 4:4′-diaminodiphenylsulphone, but some are almost as active and considerably less toxic.

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CXLVI. STUDIES IN SYNTHETIC IMMUNOCHEMISTRY

II. SEROLOGICAL INVESTIGATION OF *O*-β-GLUCOSIDOTYROSYL DERIVATIVES OF PROTEINS

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In the first paper of this series [Clutton *et al.* 1937] a method was described by which simple carbohydrate residues could be introduced into a protein in the form of the *O*-β-glycoside of tyrosine, the latter being coupled in peptide linkage with the free amino groups of the protein molecule. Application of this method to the preparation of *O*-β-glucosidotyrosylgelatin was described and it was stated that a preliminary study of the product gave no indication of the possession of antigenic properties.

The present communication deals with the extension of this work to the preparation of *O*-β-glucosidotyrosyl derivatives of other proteins and with a detailed serological investigation of the products; the possession of these new derivatives has made possible a reinvestigation of the serological properties of the gelatin compound itself, as a result of which the previous suggestion that it was non-antigenic has to be modified.

The additional proteins selected for study were (*a*) insulin, (*b*) horse serum albumin and (*c*) horse serum globulin. The case of insulin presents features of general interest in common with that of gelatin. As was pointed out in our previous paper one of our main objects was to try to discover whether the reason for the generally accepted lack of antigenic power of gelatin might be the absence from its molecule of either carbohydrate or tyrosine or both; such a possibility seemed not unlikely in view of the known importance of both tyrosine and carbohydrate groups in immunological reactions. It is, however, doubtful whether it is fair to regard gelatin as a true protein and the possibility remains that the process of its preparation from collagen involves some destructive action which diminishes or abolishes antigenic capacity; in insulin, on the other hand, we have a compound which may be regarded as a complete protein in so far as its molecular weight and amino-acid composition are concerned; as is well known, however, insulin is non-antigenic and it differs chemically from proteins which are known to be antigenic in containing no carbohydrate. It was therefore of particular interest to examine whether the introduction of glucose residues into insulin would yield an antigenic substance.

The derivatives of the serum proteins were prepared with the primary intention of ascertaining how powerfully determinant in an immunological sense the glucosidotyrosyl residue might be, i.e. to what extent the original immunological specificity of the proteins was masked by its introduction. In our previous paper we made a general criticism of earlier work on artificial protein-carbohydrate complexes on the ground that these contained an azo linkage which was foreign

to nature and which might itself affect the immunological reactions; it was hoped that a study of antisera against our glucosidotyrosyl derivatives of serum proteins in comparison with antisera against glucosidophenylazo derivatives of the same proteins might also indicate whether or not our criticism was justified.

As will be seen below, information has been forthcoming on all these points; moreover, the serum protein derivatives have proved unexpectedly useful in elucidating the true situation with regard to the derivatives of gelatin and insulin.

Experimental

Preparation of compounds

(1) O-*β-Glucosido*-N-*carbobenzyloxytyrosylgelatin*. This was prepared as described by Clutton *et al.* [1937].

(2) O-*β-Glucosido*-N-*carbobenzyloxytyrosylglobulin*. Serum obtained by sedimentation from 8 litres of defibrinated horse blood was diluted with an equal volume of water and the solution treated slowly with an equal volume of saturated aqueous $(NH_4)_2SO_4$; next day the precipitated globulin was separated by decantation and filtration, washed on the filter with half-saturated aqueous $(NH_4)_2SO_4$, suspended in a minimum of water and dialysed exhaustively against running tap water. The necessary amount of NaCl was added to bring the euglobulin into solution and the resulting 15% solution of protein was stored at 0° with thymol as preservative.

For the coupling, 10 ml. of stock globulin (1·5 g. protein) were made just alkaline to phenolphthalein and diluted to 15 ml. To this solution, cooled in ice and stirred, was added during the course of about 15 min. glucosidocarbobenzyloxytyrosylazide (from 1 g. of the hydrazide: 40% excess) in dioxane (5 ml.); constant alkalinity was maintained by simultaneous addition of 0·1 *N* NaOH.

The reaction mixture (final vol. 30–40 ml.) was diluted to 150 ml. with water and treated with 10% acetic acid to *p*H 5·0, together with a little salt; after some hours the precipitate was collected at the centrifuge and dissolved in the minimum of dilute NaOH. The solution was dialysed against running tap water for 18 hr.; traces of insoluble matter were removed by centrifuging at low speed.

The opalescent solution so obtained contained, on a nitrogen basis, an 80–90% yield of the coupled product; the glucose : nitrogen ratio indicated the introduction of 6% of glucose. The new product was insoluble in water at *p*H $< 6{\cdot}0$ but readily soluble at *p*H $> 7{\cdot}0$; it could be denatured by heat.

(3) O-*β-Glucosido*-N-*carbobenzyloxytyrosylalbumin*. The albumin was crystallized from the $(NH_4)_2SO_4$ mother liquors of the above globulin preparation by adjustment to *p*H 5·2 (approx.) with *N*/5 H_2SO_4 followed by careful addition of solid $(NH_4)_2SO_4$ to the stirred solution until crystals began to appear; the albumin was collected, recrystallized once, again collected, dissolved and dialysed free from salt, yielding a stock solution containing 10% protein which was preserved at 0° with thymol.

6 ml. of the stock albumin solution were diluted to 20 ml., made just alkaline to phenolphthalein and mixed with 20 ml. of dioxane; the mixture was then treated as above with a dioxane solution of glucosidocarbobenzyloxytyrosylazide from 0·3 g. of the hydrazide; the product was isolated from the reaction mixture exactly as in the case of the globulin derivative.

The yields were not quite so good as in the latter case, but the degree of coupling was more satisfactory, successive preparations having glucose contents of 11·5 and 11·8% respectively.

The product differs from the globulin derivative in being soluble at *p*H $<3{\cdot}8$; it has a zone of insolubility extending from *p*H 3·8 to 5·5.

(4) *O-β-Glucosido-N-carbobenzyloxytyrosylinsulin.* Crystalline insulin (Boots: 1 g.) was dissolved in water (20 ml.) plus the necessary amount of *N*/10 HCl. *o*-β-Glucosido-*N*-carbobenzyloxytyrosylhydrazide (0·7 g.) was converted into the azide as usual and this, after thorough washing with cold water, was dissolved in chilled dioxane.

The chilled and mechanically stirred insulin solution was then treated with *N*NaOH drop by drop until definitely alkaline to cresol red; the dioxane solution of the azide was added slowly, stirring being continued and alkalinity to cresol red being maintained by addition of NaOH as necessary. Stirring in the cold was continued for about 15 min. after the addition of the reagents. The solution was first diluted with dioxane to make 50 % of the latter and then neutralized; a sticky precipitate separated but the mother liquor still contained much material which was precipitated by dilution with water but was soluble in acid. The whole was therefore diluted with much water, treated with dilute HCl to produce a clear solution, and then with saturated sodium acetate to the point of optimum flocculation.

Numerous attempts were made to crystallize the product by the method employed by Scott [1934] in the case of insulin; these attempts met, however, with no success. The material was therefore purified by re-solution in water with the aid of the minimum amount of ammonia followed by re-precipitation with HCl; optimum flocculation occurred at about *p*H 5·8.

The resulting product was separated at the centrifuge, washed with water and dried in a vacuum desiccator; it formed a cream-coloured amorphous powder having solubilities very similar to those of insulin itself.

The glucose : nitrogen ratio indicated 11·0 % of coupled glucose.

Removal of carbobenzyloxy residues from glucosidocarbobenzyloxytyrosylglobulin. The method of reduction with sodium in liquid ammonia, adopted with success in the case of gelatin, failed with the globulin derivative owing to the insolubility of the latter. Recourse was therefore had to catalytic reduction.

It was first shown that the carbobenzyloxy residue could in fact be eliminated by catalytic reduction in alkaline solution in presence of globulin. Thus a mixture of 5 ml. of a solution containing 0·1 g. globulin and 0·1 g. carbobenzyloxyglutamic acid with 10 ml. of a glycine-NaOH buffer solution of *p*H 9·6 was shaken in an atmosphere of hydrogen with 0·1 g. palladium black for 75 min.; the CO_2 evolved from the reaction mixture on acidification was found by titration to be 13·2 mg. as against a theoretical amount of 14·6 mg.

A sample of the globulin derivative, after reduction by a similar process, yielded 2·2 mg. CO_2 (calc. 3·0 mg.). The best evidence of the removal of the carbobenzyloxy residues, however, is afforded by the results of electrometric titration of the reduced product (see below); the material used in this experiment was prepared as follows. The globulin derivative (0·5 g.) was reduced with hydrogen and palladium black in glycine buffer solution as described above; the catalyst was removed by centrifuging and the protein isolated from the mother liquor by precipitation with acetic acid; re-solution and re-precipitation were repeated several times to remove glycine; finally the solution of the reduction product was dialysed successively against tap water, distilled water and *N*/100 acetate buffer, *p*H 5·2, containing *N*/10 KCl.

The glucose content of the reduction product (5·6 %) was little different from that of the starting material (6·0 %).

The reduction product was titrated electrometrically by the technique of

Harington & Neuberger [1936]; as in the case of glucosidotyrosylgelatin [Clutton *et al.* 1937] the result of the titration indicated that coupling had occurred mainly with the α-amino groups of protein.

Removal of carbobenzyloxy residues from the other protein derivatives was not attempted since, as will appear later, such residues are of little, if any, immunological importance.

Serological experiments

Technique of immunization. Rabbits of 2–3 kg. weight were used, and unless otherwise stated the following injections were given at 2-day intervals:

Week I: 10 mg. intravenously three times.
Week II: 20 mg. intravenously three times.
Week III: 30 mg. intraperitoneally three times.
Week IV: 40 and 50 mg. intraperitoneally three times.

The animals were rested for 10 days and test bleedings were made.

The following derivatives were used:

(*a*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylgelatin (3 animals).

(*b*) *O*-β-Glucosidotyrosylgelatin (3 animals). An additional course of injections was given consisting of three of 30 mg. intraperitoneally and three of 50 mg. After 10 days' rest a second bleeding was made.

(*c*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylglobulin (3 animals).

(*d*) *O*-β-Glucosidotyrosylglobulin (3 animals).

(*e*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylalbumin (2 animals). In this case the last two doses of 40 and 50 mg. respectively were omitted.

(*f*) *O*-β-Glucosido-*N*-carbobenzyloxytyrosylinsulin (3 animals). These animals received a 3 weeks' course consisting of nine doses of 10 mg.

As this derivative has practically the same activity as insulin itself, each rabbit received by stomach tube, before its injection, 50 ml. of a mixture of 10 % glucose, 10 % casein, 10 % starch and 70 % water. With this precaution in no case did any of the rabbits exhibit symptoms of hypoglycaemia.

Serological tests. The antisera obtained as above were titrated against the various antigens, the interfacial ring technique with undiluted serum being employed throughout; the specificity of the reactions was studied by means of inhibition tests with appropriate compounds.

Results

Globulin derivatives. Both *O*-β-glucosido-*N*-carbobenzyloxytyrosylglobulin and its reduction product gave strong precipitation reactions up to 1 : 100,000 with the homologous antisera, the original globulin specificity being almost completely masked (Table I A, B).

Table I

	Antigen	Antiserum	Serum no.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
A.	G.C.T. globulin*	G.C.T. globulin	1	+ + +	+ + +	+ + +	+ +
	,,	,,	2	+ + +	+ + +	+ + +	+ +
	,,	,,	3	+ + +	+ + +	+ + +	+ +
	Normal globulin	,,	2	+	−	−	−
B.	G.T. globulin*	G.T. globulin	1	+ + +	+ + +	+ +	+
	,,	,,	2	+ + +	+ + +	+ +	+
	,,	,,	3	+ + +	+ + +	+ +	+
	Normal globulin	,,	2	(1/150) +	?	?	?

* In this and succeeding tables G.C.T. = *O*-β-glucosido-*N*-carbobenzyloxytyrosyl, and G.T. = *O*-β-glucosidotyrosyl.

The above precipitations at antigen dilutions of 1 : 10^5 were almost completely inhibited by preliminary incubation of the antiserum with an equal volume of 1 % glucosidocarbobenzyloxytyrosine for 15 min. at 38°.

Albumin derivative. This compound gave a powerful and highly specific reaction with its antiserum, the original albumin specificity being completely masked (Table II A). Inhibition tests with various compounds are shown in Table II B, and discussed below.

Table II

A. Precipitation tests

Antigen	Antiserum	Serum No.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
G.C.T. albumin	G.C.T. albumin	1	+ +	+ ±	+ +	+
,,	,,	2	+ +	+ + +	+ +	+
Normal albumin	,,	2	–	–	–	–

B. Inhibition tests

1 vol. antiserum—G.C.T. albumin incubated for 30 min. at 38° with 1 vol. of: *A*, glucosidocarbobenzyloxytyrosine; *B*, glucosidotyrosine; *C*, arbutin; *D*, salicin; *E*, maltose; *F*, saline each in 1 % solution.

Dilution of antigen	Antigen *A*	*B*	*C*	*D*	*E*	*F*	Time of reading min.
1 : 10^3	–	–	.	.	.	+	0
	–	–	.	.	.	+ +	15
	–	–	.	.	.	+ +	30
1 : 10^4	–	–	±	–	+ +	+ +	0
	–	–	±	–	+ +	+ +	15
	–	–	±	–	+ +	+ +	30
	.	.	–	–	+ +	+ + +	45
1 : 10^5	.	.	–	–	±	+	0
	.	.	–	–	+	+	15
	.	.	–	–	+	+ ±	30
	.	.	–	–	+	+ ±	45

Gelatin derivatives. Even after repeated courses of injection the antisera raised against *O*-β-glucosidotyrosylgelatin or its carbobenzyloxy derivative failed to give any sign of precipitation with the homologous antigens. In view of the work of Hooker & Boyd [1933] it was decided to test for precipitin reactions between the anti-gelatin derivative sera and the globulin derivative; Table III A, B, C, shows the positive results which were obtained in this case.

Table III

	Antigen	Antiserum	Serum No.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
A.	G.C.T. globulin	G.C.T. gelatin	1	+ +	+	–	–
	,,	,,	2	–	–	–	–
	,,	,,	3	±	±	–	–
B.	G.T. globulin	G.T. gelatin	1	+ ±	+	–	–
	,,	,,	2	–	–	–	–
	,,	,,	3	±	±	–	–
		Antigen controlled with normal serum negative					
C.	G.C.T. globulin	G.T. gelatin	1	+ +	+	–	–
	,,	,,	2	+	±	–	–
	,,	,,	3	+ + +	+ + +	±	–
	,,	Normal serum	Normal serum	–	–	–	–
D.	G.T. gelatin	G.C.T. globulin	1	?	+	+	?
	,,	,,	2	0	+	+	±

Table III D also shows that the reverse cross-reaction can be demonstrated, i.e. that the gelatin derivative will react with antiserum raised against the globulin derivative.

Insulin derivative. Like the gelatin compounds this substance failed to precipitate with its own antiserum; the antiserum however gave precipitation with the corresponding globulin derivative, and the globulin antiserum precipitated with the insulin derivative (Table IV A, B).

It was found, however, that the insulin derivative was capable of inhibiting the cross-reaction of its antiserum with the globulin derivative (Table IV C).

Table IV

	Antigen	Antiserum	Serum No.	Dilutions of antigen 1 : 10^2	1 : 10^3	1 : 10^4	1 : 10^5
A.	G.C.T. globulin	G.C.T. insulin	1	±	±	±	−
	,,	,,	2	+	±	+	−
	,,	,,	3	+	+	±	−
	,,	Normal serum	.	−	−	−	−
B.	G.C.T. insulin	G.C.T. globulin	.	+ + ±	+ +	+ +	+

C. Inhibition tests

Antiserum—G.C.T. insulin incubated 40 min. at 38° with (*a*) 1% insulin, (*b*) 1% G.C.T. insulin.

Inhibitor	Antigen—G.C.T. globulin, 1 : 10^3	Time min.
Insulin	+	10
	+ ±	20
G.C.T. insulin	−	10
	±	20

Discussion

Albumin and globulin derivatives

The first point of importance which emerges from the serological experiments is the powerfully determinant nature of the glucosidotyrosyl residue. This is shown clearly by the experiments recorded in Tables I and II; both globulin and albumin derivatives were strongly antigenic but neither the antiserum against glucosidocarbobenzyloxytyrosylglobulin nor that against the corresponding albumin derivative gave significant precipitin reactions with the original proteins. These results may be contrasted with those of Avery & Goebel [1929] who observed considerable residual globulin specificity when working with glucosidophenylazoglobulin.

The determinant effect of the glucosidotyrosyl residue is shown most clearly in the case of the albumin derivative, as is to be expected from the higher degree of coupling which was attained with this protein; Table II A shows that whilst good precipitin reactions are obtained between the antiserum and the coupled protein up to a dilution of 1 : 10^5 of the latter, no precipitation whatever is obtained with unaltered albumin.

The inhibition reactions shown in Table II B are of considerable significance; it will be observed that almost complete inhibition of the precipitation is obtained with four compounds which possess in common the β-phenolic glucoside linkage; inhibition with maltose on the other hand, which is a β-glucoside of different type, is very slight if indeed it exists at all. The conclusion to be drawn from this is that the determinant effect of the glucosidotyrosyl residue is conditioned entirely by the phenolic glucoside linkage. The predominant character of this

linkage is further emphasized by experiments (not recorded in detail) which have been made with a glucosidophenylazoalbumin derivative prepared by the method of Goebel & Avery [1929]; immunization with this compound yielded an antiserum with a good titre against the homologous antigen but showing marked precipitation with unaltered albumin; it gave good precipitation also with glucosidocarbobenzyloxytyrosylalbumin; the reaction between the antiserum and glucosidophenylazogelatin as well as that between the antiserum and glucosidotyrosylalbumin was completely inhibited by a β-phenolic glucoside (arbutin).

It appears therefore that, at least in the case of powerfully determinant groups, our original criticism of the azo method of coupling with proteins was ill-founded; on the other hand it can be claimed that our "peptide" method has the great advantage of completely masking the original specificity; moreover, the indications are that the carbobenzyloxy residues play little or no part in the immunological reactions.

Gelatin and insulin derivatives

As already stated, no precipitin reactions have at any time been obtained between antisera raised against the gelatin derivatives and the homologous antigens. It is, however, possible to demonstrate indirectly the presence of specific antibodies in such antisera; thus two out of three antisera against glucosidocarbobenzyloxytyrosylgelatin gave good precipitation with the analogous globulin derivative (Table III A) and, of antisera against glucosidotyrosylgelatin, two out of three precipitated with glucosidotyrosylglobulin (Table III B) and all three with glucosidocarbobenzyloxytyrosylglobulin (Table III C). These results are analogous with those recorded by Hooker & Boyd [1933] for a diazoarsanilic acid-gelatin complex.

Furthermore the reverse reaction can be demonstrated; thus from Table III D it is seen that both gelatin derivatives will precipitate with antiserum against glucosidocarbobenzyloxytyrosylglobulin; here again there exists an analogy in the experiments of Hopkins & Wormall [1933] with phenylcarbamido derivatives of gelatin and other proteins.

A precisely similar situation exists with the insulin derivative (Table IV A, B), but here the additional fact has been observed that the insulin derivative is itself able to inhibit the reaction between the anti-insulin derivative serum and glucosidocarbobenzyloxytyrosylglobulin.

Although, as has been pointed out, analogies for each of the separate types of cross-reaction just described are to be found in the literature, the combination of both types is not easy to understand. The failure to obtain direct precipitation between anti-gelatin or anti-insulin derivative serum and the appropriate homologous antigen might be explained by the assumption that the physical properties of these antigens are not such as to enable them to form an insoluble precipitate with the antibody; the validity of this assumption is, however, rendered questionable by the second type of reaction, since here the gelatin (or insulin) derivative does in fact form a precipitate with an antibody which, according to existing theories, should not differ greatly in physical properties from the antibody formed against the corresponding globulin derivative.

Whatever may ultimately turn out to be the explanation of this curious phenomenon there seems to be no escape from the conclusion that both gelatin and insulin, when coupled with glucosidotyrosine, acquire the power of inducing antibody formation, even though this can only be indirectly demonstrated; moreover, the inhibition experiments recorded in Table IV B show that the

reactions of the antibodies formed are specifically determined by the glucosidotyrosyl group which is acting as a hapten. It therefore seems reasonable to regard the glucosidotyrosyl derivatives of gelatin and insulin as complete antigens. Further evidence in favour of the antigenic effect of the insulin derivatives is afforded by the results of (as yet unpublished) anaphylactic experiments carried out by Dr S. D. Elliott in the Department of Pathology, Cambridge.

Summary

1. The preparation is described of *O*-β-glucosido-*N*-carbobenzyloxytyrosyl derivatives of horse serum albumin and globulin and of insulin.

2. The above compounds, together with the corresponding gelatin derivative, have been studied immunologically.

3. The globulin and albumin derivatives are powerful antigens; the specificity of the original protein is entirely masked and the new specificity is conditioned exclusively by the β-phenolic glucoside linkage.

4. Antisera against the gelatin and insulin derivatives fail to precipitate with the homologous antigens; they precipitate, however, with the derivative of globulin, and antiserum against the latter precipitates with the gelatin and insulin compounds themselves. It is concluded that coupling with glucosidotyrosine converts both gelatin and insulin into full antigens.

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CXLVII. STUDIES IN SYNTHETIC IMMUNOCHEMISTRY

III. PREPARATION AND ANTIGENIC PROPERTIES OF THYROXYL DERIVATIVES OF PROTEINS, AND PHYSIOLOGICAL EFFECTS OF THEIR ANTISERA

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This communication deals with the application of the general method devised by Clutton *et al.* [1937] to the preparation of thyroxyl derivatives of various proteins, and with the serological study of the products. It has also proved of interest to investigate the pharmacological properties of the antisera raised against the thyroxylproteins.

Owing to the great insolubility of thyroxine and its derivatives the direct introduction of thyroxine into the protein molecule is hardly a practicable proposition; the difficulty can be overcome, however, by proceeding in a stepwise manner. 3:5-Diiodothyronine methyl ester can readily be converted into the *N*-carbobenzyloxy derivative; this yields by the usual methods in turn the hydrazide and azide and the latter can be coupled with a protein in aqueous dioxane solution at *p*H 8·9. The *N*-carbobenzyloxy-3:5-diiodothyronylprotein thus obtained is then treated with iodine in ammoniacal solution, which converts the 3:5-diiodothyronyl residues into thyroxyl residues and at the same time converts the tyrosine of the original protein into 3:5-diiodotyrosine.

The method outlined above has been applied to the preparation of thyroxyl derivatives of horse serum albumin and globulin and of ox and human thyroglobulins. As will be seen from the results of the serological study reported below these products are all powerfully antigenic, the specific nature of their serological reactions being, as is to be expected, largely conditioned by the thyroxyl groups which have been introduced; moreover, the antisera react not only with artificial thyroxine-containing proteins but in some cases at least with thyroglobulin itself. The latter observation raises the question as to whether such antisera exercise an antagonistic effect against thyroglobulin or circulating thyroid hormone in the intact animal. The final portion of the present paper deals with the experiments which have been made to test this possibility and it will be seen that evidence has been obtained that a state of resistance to the normal physiological effect not only of thyroglobulin but of thyroxine itself can be produced in an animal by passive immunization with antiserum raised against a thyroxylprotein.

Experimental

I. *Preparation of compounds*

N-Carbobenzyloxy-3:5-diiodothyronine methyl ester. 4·2 g. diiodothyronine methyl ester [Ashley & Harington, 1928] were suspended in 80–100 ml. of anisole and 1·32 g. of benzylcarbonyl chloride were slowly added. After a few seconds' shaking 40 ml. of a solution containing 1·6 g. $NaHCO_3$ were added, and the

whole was shaken vigorously until the evolution of CO_2 had ceased. A further addition of 1·32 g. of the chloride was then made, followed by 1·6 g. of $NaHCO_3$ (40 ml.), and shaking was continued until the ester had passed completely into solution.

The anisole layer was separated and the aqueous phase washed with chloroform. The anisole and chloroform solutions were combined and evaporated under reduced pressure to a syrup which was removed from the flask with a minimum of chloroform and precipitated with light petroleum. After a few hours in the ice-chest the oily mass hardened to a yellow granular powder which was collected and dried. The yield was 4 g.

The product was insoluble in water and light petroleum, fairly soluble in chloroform, benzene and hot alcohol, and very soluble in anisole. A sample recrystallized from hot benzene by slow cooling formed elongated prisms, M.P. 164·5°. (Found: C, 42·8; H, 3·14; I, 37·2 %. $C_{24}H_{21}O_6NI_2$ requires C, 43·2; H, 3·12; I, 37·8 %.)

N-*Carbobenzyloxydiiodothyronyl hydrazide.* 4 g. of the carbobenzyloxy ester were suspended in 20 ml. of absolute alcohol and treated with 1·36 g. of pure hydrazine hydrate. The solid was dissolved by heating on a steam bath and the solution transferred to an incubator at 38°. After 2 hr. a crystalline mass had started to appear; the mixture was again brought to the boil and returned to the incubator for 12 hr.; it was then diluted with 10 ml. alcohol and kept for 24 hr. in the ice-chest. The product, consisting of large prisms, was collected, washed with alcohol and ether, and dried. Yield 4 g.

The hydrazide was insoluble in alcohol, water and ether, but soluble in pyridine and dioxane. After recrystallization from pyridine and methyl alcohol the compound had M.P. 141°. (Found: N, 6·23; I, 38·0 %. $C_{23}H_{21}O_5N_3I_2$ requires N, 6·24; I, 37·9 %.)

N-*Carbobenzyloxydiiodothyronyl azide.* 1 g. of the hydrazide was dissolved with heating in a mixture of 12 ml. glacial acetic acid, 8 ml. water and 0·3 ml. conc. HCl, and the clear solution was cooled to 0°. 1 ml. of 10 % aqueous $NaNO_2$ was added drop by drop with vigorous mechanical stirring. After 5 min. the azide had partially separated as an amorphous mass; precipitation was completed by slow dilution with 30 ml. of ice-cold water, and after a few minutes the mixture was centrifuged; the azide was washed twice with 30 ml. of ice-cold water and taken up in 15 ml. dioxane, the solution being kept as cool as possible until required for coupling.

N-*Carbobenzyloxydiiodothyronylglobulin.* Stock horse serum globulin solution containing 1·5 g. protein [cf. Clutton *et al.* 1938] was diluted with water and dioxane to make a final concentration of 1·5 % in 50 % dioxane, and the mixture was made just alkaline to phenolphthalein and cooled to 0°; a dioxane solution of 1 g. of the azide was added over 20 min. to the cooled and stirred protein solution, the alkalinity (*p*H 8–9) being maintained by addition of 2*N* NaOH.

The solution was then acidified to *p*H 5 with 10 % acetic acid, and kept for 30 min. in the ice-chest. The flocculus was centrifuged off and purified by twice or thrice repeated solution in 150 ml. of 50 % methyl alcohol with the minimum of alkali, followed by precipitation with acetic acid. Some preparations, through partial denaturation, failed to dissolve completely in 50 % methyl alcohol; in such cases 50 % alkaline dioxane was employed instead. The product was finally dissolved in 100 ml. of alkaline 50 % methyl alcohol and analysed for nitrogen and iodine (Table I).

The compound was then flocculated with acetic acid and preserved in the ice-chest pending the next step.

Iodination of N-carbobenzyloxydiiodothyronylglobulin. In this step the aim was to iodinate exclusively in the positions *ortho* to the OH-groups of tyrosine and diiodothyronine. The theoretical quantity of iodine required was therefore calculated from the tyrosine content of the original globulin (6·8 %) and the quantity of diiodothyronine introduced, a 10 % excess being allowed.

The diiodothyronylglobulin from 1·5 g. serum globulin was dissolved in 180 ml. of strong ammonia and the solution cooled to 0°. 2·72 *N* iodine solution was then run in, drop by drop, with mechanical stirring. Fresh quantities of iodine were not added until all traces of nitrogen iodide had disappeared. At the theoretical end point the reaction became sluggish and when the 10 % excess had been added the whole turned to a grey-brown colour. In later experiments the diiodothyronylglobulin was brought into solution in ammoniacal dioxane and ammonia was added until the concentration of dioxane was 10 %. (By this variation of technique better solution and iodination of the proteins were obtained.)

After 10–15 min. the opalescent solution was precipitated by pouring into a slight excess (1200 ml.) of ice-cold 10 % acetic acid. A few drops of sodium metabisulphite were then added to remove any unchanged iodine. After several hours at 0° the supernatant liquid was removed, first by decantation and finally on the centrifuge.

The precipitate was washed twice with 400 ml. of water and made acid to Congo red, and then dispersed in the same quantity of water just alkaline to phenolphthalein. Precipitation and washing were repeated as before. Finally the product was treated with 100 ml. of 30 % alkaline methyl alcohol, when solution generally occurred. The whole was precipitated with 10 % acetic acid and treated with 200 ml. of water alkaline to phenol red. The product formed a thick opalescent solution. Iodine and nitrogen determinations were made (see Table I). (Earlier preparations were not subjected to treatment with 30 % methyl alcohol and were obtained as coarse suspensions.)

N-Carbobenzyloxythyroxylalbumin. This was prepared from crystalline horse serum albumin [cf. Clutton *et al.* 1938] and the procedure was the same as that employed with the corresponding globulin derivative, except for the following modifications.

(1) After the coupling, reprecipitation was carried out once from 20 to 30 % methyl alcohol; the product was then found to be soluble in water at *p*H 8 and water was therefore used in place of aqueous alcohol for purification.

(2) The coupled product was extremely soluble in concentrated ammonia and iodination was done in an ammoniacal solution containing 2 % protein. The tyrosine content was erroneously taken as 4·7 %, but as iodination was rapid and the end point sharp, iodine was added until the mixture assumed a permanent grey-brown colour. This involved the addition of as much as 20 % excess over that required for complete iodination of the 6·3 % tyrosine actually present [Holiday, 1936].

The properties of the albumin derivative were in striking contrast to those of the globulin and thyroglobulin complexes. This product was readily soluble in water at *p*H $>8{\cdot}0$ and $<3{\cdot}9$; in acid solutions, however, traces of salt induced precipitation.

N-Carbobenzyloxythyroxylthyroglobulin. Thyroglobulin was prepared from frozen ox thyroids and from frozen normal human thyroids; the latter were obtained *post mortem* from patients dying of diseases not involving the thyroid. The thyroglobulin was isolated by the method of Harington & Salter [1930] and was purified by two reprecipitations.

The coupling with *N*-carbobenzyloxydiiodothyronine and the subsequent iodination were conducted exactly as in the case of serum globulin, the tyrosine content of the thyroglobulin being assumed to be 5·4 % [Eckstein, 1926]. The product formed a much more stable solution than did the serum globulin derivative and in several preparations the final treatment with 30 % methyl alcohol could be omitted.

The analytical data relating to the various preparations of thyroxylproteins are contained in Table I.

Table I. *Analytical data regarding thyroxylproteins*

		Coupled solution		Iodinated solution	
Protein g.	Hydrazide g.	mg. N per 100 ml.	mg. I per 100 ml.	mg. N per 100 ml.	mg. I per 100 ml.
		A. *N*-Carbobenzyloxythyroxylglobulin			
1·5	1·0	230	235	—	—
1·5	1·0	232	188	182	355
1·5	1·0	224	204	172	290
0·87	0·5	252	147	179*	303
		B. *N*-Carbobenzyloxythyroxylalbumin			
0·48	0·4	252	206	174	391
2·0	1·2	370	240	246	382
		C. *N*-Carbobenzyloxythyroglobulin			
		Thyroglobulin A_1–Ox, contained 0·5 % I. Thyroglobulin A_2–Ox, contained 1·0 % I. Thyroglobulin B–Man, contained 0·5 % I			
2·5 A_1	2·0	190	96	144	155
1·5 A_2	0·9	114	100	120*	252
1·5 B	1·0	239†	294†	220*	462

* Iodination carried out in a mixture of dioxane (1 part) and conc. ammonia (9 parts).
† Poor reprecipitation.

The following additional compounds were prepared for use in the serological tests.

Iodoglobulin. Horse serum globulin (10 ml. of 15 % solution) was mixed with concentrated ammonia (20 ml.; sp. gr. 0·880) and the whole treated at 0° with iodine (0·95 ml. of 2·72 *N*); further treatment was exactly as described above for thyroxylglobulin. The product contained about 6·0 % iodine; it still gave a faint Millon reaction.

Tryptic digest of thyroglobulin. About 60 g. of ox thyroglobulin were heat-coagulated and suspended in 400 ml. of water together with 10 ml. of 2 *N* HCl. 0·5 g. of pepsin (Parke Davis 1 : 10,000) was added and the whole kept for 48 hr. at 38°. Another 0·5 g. of pepsin was added and digestion continued for 48 hr. longer. At this stage most of the protein was in solution; the whole was brought to *p*H 8 with 2 *N* NaOH and treated with 0·5 g. trypsin (Merck).

After 48 hr. the solution was acidified to Congo red, the precipitate which formed being centrifuged off and washed with 500 ml. water. The dark brown sediment was suspended in 300 ml. of water and brought to *p*H 8. The whole was re-digested with 1·5 g. trypsin for 36 hr. at 38°, and with a further 1·5 g. trypsin for 48 hr. longer.

At this stage the amino-N amounted to 38 % of the total N.

The solution was again brought to Congo red point with HCl, the precipitate spun off, washed several times with 50 % acetone and finally with dry ether. Yield 6·0 g. Iodine 5·6 %.

The product was made up in 1 % solution and used for inhibition experiments.

II. *Immunological experiments*

In all cases 3 injections were given per week unless severe loss of weight necessitated a short rest. In all cases the rabbits were bled from 8 to 10 days after the completion of their course of injections.

(1) *Antigen thyroxyl-serum globulin.* Two groups of rabbits were used. The first group received 4 doses of 10 mg., 2 of 20 and 5 of 30 mg., followed by a month's rest, then one further dose of 20 mg. and 7 of 30 mg. Two rabbits in this group of three died, one from protein shock and one from excessive loss of weight.

The second group received 3 doses of 10 mg., 3 of 20 and 3 of 30 mg. All remained well during the course.

(2) *Antigen thyroxylalbumin.* One group of rabbits was used, which received 3 doses of 10 mg., 3 of 20 and 3 of 30 mg. All the rabbits lost weight and one of the group died from excessive loss of weight.

(3) *Antigen thyroxylthyroglobulin.* Three groups of rabbits were used. Each group received 3 doses of 10 mg., followed by 3 doses of 20 mg. and then 3 doses of 30 mg. Two of the groups received 4 and 5 additional doses respectively, after a week's rest, the doses varying from 30 to 50 mg.

Two of these rabbits died from protein shock and nearly all lost weight. The most severe loss of weight amounted to about a quarter of the original body weight.

Certain of the antigens used were in the form of suspensions and were unsuitable for precipitin tests. Two techniques could be used to obtain them as solutions.

(*a*) *Pyridine technique.* 20 mg. of the antigen were completely precipitated with acetic acid. The precipitate was centrifuged off and dissolved in 1 ml. of

Table II. *Precipitin reactions with antiserum from rabbit No. 38 prepared against thyroxylglobulin*

Antigen	Antiserum	Dilutions of antigen 1/100	1/500	1/1000	1/10,000	1/100,000	Time of reading min.
Thyroxylglobulin (pyridine)	38	.	+ + + +	+ + +	+ +	+	60
	Normal serum	.	+ + +	±	−	−	60
Thyroxylthyro-globulin (pyridine)	38	.	+ + + +	+ +	+ + +	+ +	90
	Normal serum	.	+ ±	±	−	−	90
Iodoglobulin	38	+ +	.	+ + +	+ +	0	60
Thyroxylalbumin	38	+	.	+	+	+	60
Diiodothyronyl-albumin	38	−	.	+ ±	±	+	60
Thyroglobulin	38	−	.	+	+ ±	−	60
Albumin	38	−	.	−	−	−	60
Globulin	38	−	.	−	−	−	60

Inhibition tests

A. 1 vol. serum 38 + 1 vol. diiodotyrosine 1·0% incubated at 38° for 1 hr.
B. 1 vol. serum 38 + 1 vol. saline 1·0% incubated at 38° for 1 hr.

	Antigens: Thyroxylglobulin 1/30,000	Thyroxylglobulin 1/60,000	Thyronylalbumin 1/1000	Thyronylalbumin 1/10,000	Thyroglobulin 1/100	Thyroglobulin 1/1000
A	+	±	+	+	−	±
B	+ +	+ +	+	+	−	+ ±

Table III. *Precipitin reactions of antisera from rabbits Nos. 54 and 55 prepared against thyroxylalbumin*

Antigen	Antiserum	Dilutions of antigen 1/100	1/500	1/1000	1/10,000	1/100,000	1/1,000,000	Time of reading min.
Thyroxylalbumin	54	+ +	.	+ +	+ +	+	±	60
	55	+ + ?	.	+	+	±	−	60
	Normal serum	−	.	−	−	−	−	60
Thyroxylthyroglobulin (pyridine)	54	.	+ +	±	±	−	.	60
Thyroglobulin	54	+ + + ppt.	.	+ ±	±	−	.	60
Iodoglobulin	54	+ +	.	+ +	+	−	.	60
Diiodothyronylalbumin	54	±	.	±	+ +	+	.	60
Thyroglobulin	54	+ ?	.	−	−	−	.	60
Albumin	54	−	.	−	−	−	.	60
Globulin	54	−	.	−	−	−	.	60
Thyroxylthyroglobulin (pyridine)	Normal serum	±	.	±	−	.	.	60

Inhibition tests

1 vol. serum 54 + 1 vol. diiodotyrosine 1·0 % incubated at 38° for 1·5 hr.
1 vol. serum 54 + 1 vol. saline 1·0 % incubated at 38° for 1·5 hr.

	Thyroxylalbumin 1/100	1/1000	1/10,000	Time min.
54 + Inhibitor	?	+	+	60
54 + Saline	?	+ ±	+ +	60

Table IV. *Precipitin reactions of antisera from rabbits Nos. 50, 51 and 53 prepared against thyroxylthyroglobulin*

Antigen	Antiserum	Dilutions of antigen 1/100	1/500	1/1000	1/10,000	1/100,000	Time of reading min.
Thyroxylthyroglobulin (pyridine)	50	.	+ + +	+ + +	+ +	−	60
	51	.	+ + + +	+ +	+	−	60
	53	.	+ + + +	+	+	−	60
	Normal serum	.	+	−	−	−	60
Thyroxylglobulin	51	+ + +	.	+ +	+ +	+	60
Iodoglobulin	51	+ + + +	.	+ + ±	+	−	60
Diiodothyronylalbumin	51	+ ?	.	−	−	−	60
Thyroxylalbumin	51	+ ?	.	−	−	−	60
Thyroglobulin	51	−	.	±	+	−	60
Thyroxylalbumin	Normal serum	+ ?	.	.	.	.	60

Inhibition tests

A. 1 vol. serum 51 + 1 vol. diiodotyrosine 1·0 % incubated at 38° for 1 hr.
B. 1 vol. serum 51 + 1 vol. thyroglobulin digest 1·0 % incubated at 38° for 1 hr.
$C_1 = C_2$. 1 vol. serum 51 + 1 vol. saline 1·0 % incubated at 38° for 1 hr.

	Antigens: Thyroglobulin 1/100	Thyroglobulin 1/1000	Thyroxylglobulin 1/30,000	Thyroxylglobulin 1/60,000	Thyroxyl-thyro-globulin	Time min.
A	−	−	−	−	.	60
C_1	−	+	+	+	.	60
	1/1000	1/10,000	1/1000	1/10,000	1/10,000	
B	−	−	Tr.	Tr.	±	45
C_2	+ ±	+	+	±	+	45

Bulk tests at 56° showed that the precipitin reaction between thyroxylthyroglobulin and its antiserum was inhibited much more strongly by a mixture of thyroxine (0·75%) and diiodotyrosine (1·0 %) than by diiodotyrosine alone.

warm pyridine; the resultant solution was made up to 10 ml. with warm water, filtered and diluted with saline as required.

(*b*) *Methyl alcohol technique.* The technique was identical with the above except that the precipitate was dissolved in 1 ml. of methyl alcohol alkaline to phenolphthalein.

All tests unless otherwise mentioned were made with the interfacial ring technique. The results are given in Tables II–IV.

Discussion

The methods employed in the preparation of the compounds call for little comment; the coupling with the azide of *N*-carbobenzyloxy-3:5-diiodothyronine is exactly analogous with that of proteins with glucosidotyrosylazide described in the preceding paper. It is evident from Table I that considerable latitude in the proportion of azide to protein can be allowed without greatly affecting the result of the coupling.

The conditions chosen for iodination were designed to favour substitution with iodine and to suppress oxidation; there seems little doubt that many so-called iodinated proteins which have been used in the past have been as much oxidized as iodinated; at least, their method of preparation, involving the use of dilute alkaline solutions at ordinary temperatures, has been such as to favour the action of hypoiodite which is an effective oxidizing agent for amino-acids.

Even under careful working conditions we have ourselves been unable to obtain products of constant composition, as will appear from a study of Table I; this failure is due to difficulties of manipulation, many of the compounds having unfavourable solubility properties. Nevertheless, we have not failed in any instance to obtain a reasonably high degree of coupling with diiodothyronine and of subsequent uptake of iodine in proportion to the number of phenolic groups available.

The serological experiments have given results which are in general accordance with expectation. Thus, all the thyroxylproteins are powerful antigens (Tables II–IV). The unsatisfactory normal serum control in the case of the globulin derivative (Table II) is due to the pyridine technique which had to be employed to obtain a solution of the antigen; in spite of this the specific precipitation appears quite clearly at the higher dilutions of antigen. None of the thyroxylproteins retained any of the original protein specificity; this is to be expected in view of the drastic nature of the treatment to which they have been submitted.

The cross-reactions of the various sera and antigens are of considerable interest. Thus the antisera against thyroxylglobulin (Table II) and thyroxylthyroglobulin (Table IV) are extremely similar to one another; the thyroxylglobulin antiserum reacts as strongly with thyroxylthyroglobulin as with thyroxylglobulin itself, and the converse is true of the thyroxylthyroglobulin antiserum. Both sera also react strongly with ordinary iodoglobulin; a difference appears in the reactions of the sera with thyroxyl- and diiodothyronyl-albumin, which are fairly strong in the case of the thyroxylglobulin antiserum but weak in the case of the serum against thyroxylthyroglobulin. In accordance with this, the thyroxylalbumin antiserum (Table II), although precipitating to a high dilution with the homologous antigen, shows definitely less marked cross-reactions with thyroxylglobulin and thyroxylthyroglobulin.

One of the chief points of interest lies in the slight but definite reactions observed between thyroglobulin itself and the antisera against both thyroxylglobulin and thyroxylthyroglobulin. The question of the antigenicity of thyro-

globulin has not been satisfactorily settled. It has been known since the work of Hektoen *et al.* [1923] that preparations of thyroglobulin, when injected into a sufficiently remote species, give rise to precipitating antisera; in our opinion, however, this work has been too readily accepted as proof that thyroglobulin can itself act as an antigen. It has to be remembered that thyroglobulin is an extremely difficult protein to purify; it cannot be crystallized, and we at least have never been able to obtain preparations which we could regard as being free from tissue proteins. We are therefore inclined to ascribe the alleged precipitin formation by thyroglobulin to the presence of adventitious tissue proteins, and in this connexion the fact that precipitin formation can only be elicited between remote species seems to us to be significant. On general grounds it appears to us to be no more likely that thyroglobulin, with its specialized physiological function, should be antigenic than that insulin should be so. If this hypothesis is correct our thyroxylthyroglobulin must be antigenic by virtue of the alteration induced by the introduction of diiodothyronine residues followed by iodination, a supposition which is supported by the results of inhibition tests. Thus partial or complete inhibition of all cross reactions, including those involving thyroglobulin, is obtained with diiodotyrosine, and even more effective inhibition with a mixture of diiodotyrosine and thyroxine and with a digestion product of thyroglobulin containing thyroxine; this indicates that in all cases the determinant group is a complex involving both thyroxine and diiodotyrosine; it is particularly to be noted that there is a distinct difference between the effects of thyroxine and diiodotyrosine, suggesting that the whole of the aromatic portion of the thyroxine molecule plays a part in the immunological reactions just as it does in the physiological action of the compound; neither immunological nor physiological reactions are conditioned by the 3:5-diiodophenolic grouping alone.

Further evidence on this point is available from the facts that the antisera against thyroxylglobulin and thyroxylthyroglobulin do give slight precipitation with diiodothyronylalbumin and that this precipitation is not inhibited by diiodotyrosine; such an observation admits of no other explanation than that the 3:5-diiodophenolic ether linkage of thyroxine is involved.

It has been shown by Snapper & Grünbaum [1935] that the so-called serological reactions of thyroglobulin are not inhibited by thyroxine or diiodotyrosine; as has been pointed out above, however, these serological reactions have, in our view, probably nothing to do with true thyroglobulin and therefore the results of Snapper & Grünbaum are exactly what would be expected. Our own experiments, on the other hand, seem to prove beyond doubt that thyroxine and diiodotyrosine are capable of functioning as determinant groups in the molecule of thyroglobulin, as would be anticipated from what is known of their mode of combination in the protein and from the general importance of aromatic groups in serological reactions.

III. *Physiological experiments*

As pointed out in the introductory portion of this paper, the strongly antigenic character of the thyroxylprotein derivatives, the specific nature of their serological reactions and particularly the observation of cross-reactions between their antisera and thyroglobulin itself, raised the question of the possible inhibitory effect of such antisera on normal thyroid secretion. The method chosen to investigate this point was the observation of the effects of the antisera, alone and in combination with thyroglobulin and thyroxine, on the metabolic rate.

The experiments were carried out on young adult male rats (180–300 g. in weight) and determinations of the metabolic rate were made in the apparatus of

Richards & Collison [1928] using the technique and precautions described by Gaddum [1929–30]. The animals were kept on a constant diet and were given water only for at least 17 hr. before each observation. It was found to be advantageous to restrict the movements of the animals within the metabolism chamber by placing them in a light wire cage of such size as to moderate their activity without causing discomfort. An element of training enters into the experiment, since the animals require some time to become accustomed to the procedure; with each animal therefore a preliminary series of observations was made under normal conditions and in no case was an animal used for experiment which had not given at least two (and generally three) consecutive results of reasonable constancy.

Under the conditions employed, the basal oxygen consumption of the normal rats varied in different animals between the approximate limits of 13·5 and 16·5 ml./kg./min.; the three last readings in the pre-experimental period were averaged and the resulting figures were taken to represent the basal level; the detailed course of two typical experiments is shown in the protocols in the Appendix to this paper.

The first experiments to be made were concerned with the administration of antiserum against thyroxylthyroglobulin to normal rats; the results of these were entirely negative, no effect on the metabolic rate being observed during the (relatively short) period over which the injections were maintained.

Table V. *Effect of previous treatment with antiserum prepared against thyroxylthyroglobulin on metabolic rate of rats injected with thyroglobulin*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
A. 1	5 doses of 12 ml. anti-serum	4 doses of 5 ml. thyroglobulin (0·5% solution)	15·2
2	,, ,, ,,	,, ,, ,,	29·0
3	,, ,, ,,	,, ,, ,,	7·7
4	,, ,, ,,	,, ,, ,,	2·8
Controls			
1	5 doses of 12 ml. normal rabbit serum	4 doses of 5 ml. thyroglobulin (0·5% solution)	62·5
2	,, ,, ,,	,, ,, ,,	62·4
B. 1	4 doses of 13 ml. anti-serum	1 dose of 0·50 ml. thyroglobulin (3·5% solution)	11·3
2	,, ,, ,,	,, ,, ,,	9·1
3	,, ,, ,,	,, ,, ,,	9·1
Controls			
1	4 doses of 13 ml. normal rabbit serum	1 dose of 0·50 ml. thyroglobulin (3·5% solution)	58·8
2	,, ,, ,,	,, ,, ,,	40·8

Notes:

Rats	Serum given on days	Thyroglobulin given on days
Group A:		
1	1, 2, 3, 4, 6	8, 9, 10, 13
2	1, 2, 3, 4, 7	9, 10, 11, 14
3	1, 2, 3, 4, 5	8, 9, 10, 12
4	1, 2, 3, 4, 5	8, 9, 10, 12
Controls		
1	1, 2, 4, 5, 6	6, 7, 8, 9
2	1, 2, 3, 4, 5	7, 8, 9, 11
Group B:		
Control 2	1, 2, 3, 4	4
All others	1, 2, 3, 5	5

Table VI. *Effect of previous treatment with antiserum prepared against thyroxylglobulin on metabolic rate of rats injected with thyroglobulin*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
A. 1	3 doses of 2 ml. antiserum (concentrated 1 : 3)	1 dose of 0·5 ml. thyroglobulin (3·5% solution)	12·8
2	,, ,,	,, ,,	2·2
3	,, ,,	,, ,,	13·7
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	1 dose of 0·5 ml. thyroglobulin (3·5% solution)	44·0
2	,, ,,	,, ,,	28·6
B. 1	2 doses of 3 ml. antiserum (concentrated 1 : 3)	2 doses of 0·5 ml. thyroglobulin (3·5% solution)	18·7
2	,, ,,	,, ,,	34·0
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	2 doses of 0·5 ml. thyroglobulin (3·5% solution)	60·2
2	,, ,,	,, ,,	77·3
C. 1	2 doses of 3 ml. antiserum (concentrated 1 : 3)	1 dose of 1 ml. thyroglobulin (3·5% solution)	29·0
2	,, ,,	,, ,,	44·6
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	1 dose of 1 ml. thyroglobulin (3·5% solution)	24·0
2	,, ,,	,, ,,	26·6

Notes: Group A. Nos. 3 and 2, serum days 1, 2, 2 (evening). Thyroglobulin day 3.
No. 1, serum days 1, 2, 3. Thyroglobulin day 3 (evening).
Controls 1, serum days 1, 2. Thyroglobulin day 3.
Group B. All. Serum days 1, 2. Thyroglobulin days 3, 4.
Group C. All. Serum days 1, 2. Thyroglobulin day 2 (evening).

We then proceeded to examine the effect of previous administration of antiserum on the action of a dose of thyroglobulin. The results in so far as the antiserum against thyroxylthyroglobulin are concerned are shown in Table V. Two sets of experiments were performed; in the first the thyroglobulin was given as four doses of a dilute solution at daily intervals and in the second as a single dose of a more concentrated solution; in both sets administration of thyroglobulin was preceded by treatment with antiserum and both sets were controlled by experiments in which similar amounts of normal rabbit serum and of thyroglobulin were given in the same time relationship to one another. It is evident from the figures that the protective effect of the antiserum against the physiological action of thyroglobulin is high.

The experiments were next extended to the antiserum against thyroxylserum globulin. In this and subsequent cases the antiserum was concentrated by precipitation of the total globulin with an equal volume of saturated ammonium sulphate; the precipitate was collected, dissolved in water and dialysed exhaustively against running tap water, after which sodium chloride to 0·9% was added to bring the euglobulin back into solution. The results in Table VI show that in two sets out of three a high degree of protection against the action of thyroglobulin was obtained with this concentrated antiserum also; the failure in the third set remains unexplained; it may be pointed out however that the

controls in this set gave a quite abnormally low response to a large dose of thyroglobulin.

The general success in obtaining protection against the action of thyroglobulin encouraged us to investigate whether a similar protective effect against thyroxine could be observed. Tables VII and VIII show that such is indeed the case. With antiserum against thyroxylthyroglobulin (Table VII) four out of five experimental rats showed very marked inhibition of the action of thyroxine as compared with the controls and a similar statement applies to the experiments with antiserum against thyroxyl-serum globulin (Table VIII).

Table VII. *Effect of previous treatment with antiserum prepared against thyroxylthyroglobulin on metabolic rate of rats injected with thyroxine*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
1	3 doses of 3 ml. antiserum (concentrated 1 : 1·75)	3 doses of 0·75 mg. thyroxine	36·2
2	,, ,,	,, ,,	16·6
3	,, ,,	,, ,,	24·1
4	,, ,,	,, ,,	9·7
5	,, ,,	,, ,,	12·6
Controls			
1	3 doses of 3 ml. normal rabbit serum (concentrated 1 : 1·75)	3 doses of 0·75 mg. thyroxine	61·1
2	,, ,,	,, ,,	42·0

Note: All rats were given serum on days 1, 2, 3, and thyroxine on days 3 (evening), 4 and 5.

Table VIII. *Effect of previous treatment with antiserum prepared against thyroxylglobulin on metabolic rate of rats injected with thyroxine*

Rat no.	Pre-treatment	Dosage	Maximum % rise in metabolic rate
1	2 doses of 3 ml. antiserum (concentrated 1 : 3)	3 doses of 0·75 mg. thyroxine	14·3
2	,, ,,	,, ,,	15·9
3	,, ,,	,, ,,	19·4
4	,, ,,	,, ,,	21·9
5	,, ,,	,, ,,	52·9
Controls			
1	2 doses of 3 ml. normal rabbit serum (concentrated 1 : 3)	3 doses of 0·75 mg. thyroxine	65·8
2	,, ,,	,, ,,	42·3
3	,, ,,	,, ,,	46·0
4	,, ,,	,, ,,	30·0

Note: All rats were given serum on days 1, 2, and thyroxine on days 2 (evening), 3 and 4.

Discussion

There seems to be little doubt from the experiments which have just been described that the serological reactions recorded in the second section of this paper afford a true indication of the power of antisera against thyroxylproteins to react with and neutralize in the body exogenously administered thyroglobulin and thyroxine.

The failure to observe any effect of the antiserum upon the normal animal might be explained by a difference of the actual thyroid secretion from either thyroxine or thyroglobulin; since however these two compounds represent, so to

say, the extreme limits of simplicity on the one hand and complexity on the other which are conceivable for the composition of the thyroid secretion, and since both these compounds are physiologically neutralized by the antisera, such an explanation is unlikely. In any case the point will be put to direct test by examining whether the effect of thyrotropic hormone, which may be presumed to act by increasing the rate of output of normal thyroid secretion, is also neutralized by the antisera. It seems more likely that the failure of the antisera to affect the normal animal is due to the well-known power of the thyroid gland to respond to any call for increased secretion.

The inhibitory effect of the antisera on the action of exogenously administered thyroglobulin seems to be unequivocal, and indeed, in view of the serological results, is perhaps not surprising; more striking is the inhibitory effect on the action of thyroxine. At first sight the latter result seems unexpected, for whilst the neutralization of the protein thyroglobulin might be regarded as analogous with toxin-antitoxin neutralization, thyroxine is a low-molecular substance which would not be expected to take part in a reaction of this type. On the other hand it must be remembered that the serological inhibition reactions actually afford an analogy for the neutralization of thyroxine *in vivo*; the inhibition reactions are due to the power of thyroxine to combine with the specific antibodies and such combination occurring *in vivo* might well inhibit its physiological action.

The facts at any rate are clear and they seem to open up considerable possibilities since they suggest that immunization with a protein to which is attached a physiologically active group acting as a hapten may give rise to an antiserum with the power of neutralizing the normal physiological effect of the attached group *in vivo*. It must be pointed out that it is not safe to draw this general conclusion from the results obtained with thyroxine alone; thyroxine is an amino-acid and may have to be built up into the molecule of a protein before it can exercise its effect in the body; if such is the case it would be the thyroxine-containing protein which is neutralized by the antiserum and the reaction *in vivo* would fall into a recognized immunological category. Experiments are planned to test the validity of the general proposition by the use as hapten of a physiologically active group entirely unconnected with protein. It is also intended to attempt to produce a state of resistance to the action of thyroxine and other physiologically active groups by active immunization with proteins containing these groups as haptens.

It was anticipated at the outset of this work that the nature of the protein used as the basis for the artificial antigen might be of significance; actually, as between serum globulin and thyroglobulin, this does not seem to be the case, since antisera against derivatives of both these proteins are extremely similar both serologically and physiologically; it may be concluded from this that the iodine-containing groups introduced in the present experiments are so powerfully determinant in character as to override the effect of structural differences between the proteins used.

The preparation of large amounts of antisera of the type described in the present paper has been undertaken in order to test the possibility of their therapeutic application in human hyperthyroidism.

Summary

1. The preparation and properties of thyroxyl derivatives of horse serum globulin and albumin and of thyroglobulin are described.

2. The serological reactions of the derivatives have been studied and have been found to be conditioned by the thyroxine groups introduced which thus

act as haptens. The hapten property is a function of the whole aromatic portion of the thyroxine molecule and not of the diiodophenolic group alone.

3. Passive immunization with antisera against the thyroxylproteins protects against the normal physiological effects of exogenously administered thyroglobulin and thyroxine. The general implications of the latter observation are discussed.

The new derivatives of 3:5-diiodothyronine, described for the first time in this paper, were prepared some years ago in this Department by Dr S. Kishi. We desire to thank Messrs Hoffmann-la Roche and particularly Dr M. Guggenheim for generous gifts of 3:5-diiodothyronine; our thanks are also due to Sir Henry Dale and Prof. T. R. Elliott for the loan of the metabolism apparatus. One of us (M. E. Y.) is indebted to the Medical Research Council for a personal grant.

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APPENDIX

Protocol 1

Rat no. 1. Treatment: injections of antiserum to thyroxylthyroglobulin followed by thyroxine

Date	Time	Oxygen absorbed at N.T.P. ml./kg./min.	Injections	Weight g.
31. iii. 38	16.00	16·72	—	228
1. iv. 38	16.00	16·74	—	232
2. iv. 38	11.30	—	3 ml. antiserum (conc. 1 : 1·75) I.P.	—
3. iv. 38	11.30	—	3 ml. antiserum (conc. 1 : 1·75) I.P.	—
4. iv. 38	10.15	16·50	—	212
4. iv. 38	12.00	—	3 ml. antiserum (conc. 1 : 1·75) I.P.	—
4. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
5. iv. 38	10.15	16·89	—	210
5. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
6. iv. 38	10.15	17·16	—	210
6. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
7. iv. 38	10.20	18·84	—	205
8. iv. 38	10.00	16·70	—	203
9. iv. 38	10.15	17·36	—	202

Average of first 3 readings = base-line reading = 16·652 ml. O_2/kg./min.
Highest percentage rise after thyroxine injections = 13·1.

I.P. = intraperitoneal. I.M. = intramuscular.

Protocol 2

Rat no. 2. Control for rat no. 1. Treatment: injections of normal rabbit serum followed by thyroxine.

Date	Time	Oxygen absorbed at N.T.P. ml./kg./min.	Injections	Weight g.
29. iii. 38	10.15	17·33	——	210
29. iii. 38	16.00	—	3 ml. normal serum (conc. 1 : 1·75) I.P.	—
30. iii. 38	14.15	15·25	—	210
30. iii. 38	16.00	—	3 ml. normal serum (conc. 1 : 1·75) I.P.	—
31. iii. 38	10.30	14·68	—	208
31. iii. 38	12.00	—	3 ml. normal serum (conc. 1 : 1·75) I.P.	—
31. iii. 38	16.00	—	0·75 mg. thyroxine I.M.	—
1. iv. 38	13.55	20·19	—	200
1. iv. 38	16.00	—	0·75 mg. thyroxine I.M.	—
2. iv. 38	09.45	21·88	—	192
2. iv. 38	11.30	—	0·75 mg. thyroxine I.M.	—
3. v. 38	10.15	22·57	—	188
4. iv. 38	10.15	24·89	—	178
5. iv. 38	10.00	21·88	—	196
7. iv. 38	10.00	17·97	—	198

Average of first three readings – base-line reading = 15·752 ml. O_2/kg./min.
Highest percentage rise after thyroxine injections = 57·8.

CXLVIII. THE SYNTHESIS OF PURINES FROM HISTIDINE BY THE DOG

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The material for the synthesis of the purine ring in mammalian metabolism is still controversial. The first evidence that an exogenous amino-acid might serve as the precursor in the young rat was supplied by Ackroyd & Hopkins [1916]. They found that the removal of arginine or histidine from the diet diminished the excretion of allantoin by 40–50%. In human adults Lewis & Doisy [1918] were unable to demonstrate any effect of comparable diets, high and low in histidine and arginine, on the output of uric acid. The high content of arginine in placental protein suggested this amino-acid as the important precursor for the embryo. Hence Harding & Young [1919] attempted to distinguish between arginine and histidine by feeding placental and meat proteins to puppies. On the placental diet more allantoin and uric acid were excreted. Rose [1921, 1] has suggested that these results may be explained by assuming the presence of preformed purines in their diets. Rose & Cook [1925] have repeated the experiments of Ackroyd & Hopkins with young rats and conclude that, under ordinary conditions of diet, histidine may act as the mother-substance of allantoin and that arginine cannot replace histidine as a precursor of allantoin. More recently György & Thannhauser [1929] have failed to detect any influence on the excretion of uric acid in infants of 4–8 months from the addition of histidine to diets of milk and of a casein hydrolysate from which most of the arginine and histidine had been removed. Thus the origin of the purine nucleus in anabolism is supported by slender evidence and this is confined to one animal, the rat.

Rose [1921, 2] has further questioned the logic of expecting an increase in the output of allantoin or uric acid in response to altered levels in the intake of protein or amino-acid, and suggests that the synthesis of purines "is limited quantitatively to the anabolic needs of the organism", at least in the adult. In the young growing animal there must be a daily demand for purines, in excess of the supply made available by endogenous metabolism, to serve in the synthesis of nucleoproteins. On the basis of the law of mass action the concentration of precursors should influence the reaction producing such purines. The hypothesis involves the assumptions (1) that such precursors of exogenous origin reach the locus of synthesis, (2) that the reaction is sufficiently rapid as contrasted with other mechanisms of disposal to be measurable and (3) that excess purines so produced are subsequently converted into uric acid or allantoin. We have attempted to contrast for the puppy the metabolic effects of synthetic diets, high and low in arginine and histidine, and to determine whether the level of purine excretion could be related to the level of ingestion of histidine or arginine.

Experimental

Three mongrel puppies 4–8 weeks of age were confined in metabolism cages and the urine was collected under toluene in 24 hr. periods. Analytical estimations and methods were (1) total N, Kjeldahl; (2) creatinine, Folin microcolorimetric; (3) uric acid, Christman & Ravwitch [1932]; (4) allantoin, Larson [1932].

The diets made use of a protein preparation fed on the basis of 1 g. N per kg. body wt. to give, together with sucrose and lard, 100 Cal. per kg. [Cowgill, 1923]. The following were also incorporated in the diet per kg. body wt., 60 mg. yeast concentrate (Harris), 1 minim halibut liver oil (Abbott), 0·2 g. salt mixture [Karr, 1920], 0·4 g. bone ash. Commercial casein was selected as the standard protein for comparison. Haemoglobin was obtained from ox blood by centrifuging and washing the corpuscles with 0·9 % NaCl, laking with distilled water, precipitating with alcohol and ether and drying in warm air. An arachin-conarachin mixture was prepared from shelled peanuts by grinding, extracting several times with ether and drying in air. This meal still contained 18·4 % fat by Soxhlet determination.

The content of preformed purines was determined by a modified Krüger and Schmidt method in these proteins after hydrolysis. The values obtained were: casein 1·0, haemoglobin 9·2, peanut meal 1·4, expressed as mg. purine N per 100 g. protein stock preparation.

Since the publication of the new technique of Graff & Maculla [1935] and their criticism of older procedures we have applied their method to our stock preparations with the following results—casein, 14 mg.; haemoglobin, 43 mg.; peanut meal, 17 mg.

These values are still small enough to be of little significance if the amount of protein ingested be considered in relation to the purines excreted. Thus the daily intake of 10–20 g. protein would represent the ingestion of from 1·4 to 8·6 mg. purine N. The preformed purines would be equivalent to between 5 and 10 % of the total urinary purines and would correspond approximately to the uric acid excreted.

These diets were found satisfactory and all animals increased in weight on them although on the haemoglobin there was a loss of appetite after a few days. In Table I will be found the main constituents of the diets expressed per kg. body wt.

Table I. *Main dietary constituents per kg. body wt.*

Diet	Protein g.	Sucrose g.	Lard g.	Calories
Casein	7·94	8·06	4·00	100
Haemoglobin	6·45	9·55	4·00	100
Arachin	8·93	9·33	2·00	100

In the following Tables, II, III and IV, the daily urinary analyses or the average for a period are presented with the sequence of diets. The standard diet of casein was interposed between each special protein and the general agreement of the levels of excretion of urinary constituents on these control periods is unexpectedly close. In Table II, allantoin is 65 % higher on haemoglobin and 57 % lower on arachin than the control. In Table III allantoin is 105 % higher on haemoglobin and 95 % lower on arachin than the control. In Table IV allantoin is relatively constant in the first four periods. There is a slight increase on haemoglobin which appears to be carried over into the next control period

Table II. *Puppy M. Weight 1·9 kg.*

Date	Diet	Total N mg.	Urea + NH_3 N mg.	Creatinine N mg.	Uric acid N mg.	Allantoin N mg.
9 Feb.	Casein	820	735	10·3	2·1	63
10 „		878	765	14·7	2·1	64
11 „		955	815	13·3	2·9	57
12 „		993	867	12·5	3·8	54
13 „		1039	907	12·1	3·5	50
		937	**818**	**12·6**	**2·9**	**58**
14 Feb.	Haemoglobin	1685	1379	—	4·8	90
15 „		1541	825	12·7	4·2	96
16 „		1436	1078	12·5	3·2	78
		1554	**1093**	**12·6**	**4·0**	**88**
17–27 Feb.	Casein	**947**	**766**	**14·6**	**1·9**	**52**
28 Feb.	Arachin	577	346	12·8	1·6	55
1 Mar.		660	583	13·7	1·8	40
2 „		715	630	12·1	1·5	20
3 „		764	663	15·8	1·8	26
4 „		575	574	10·5	1·2	30
5 „		619	562	9·6	1·3	17
6 „		568	506	8·2	1·3	14
7 „		408	364	6·1	0·9	15
8 „		330	155	6·1	0·7	20
9 „		255	208	7·2	0·6	16
10 „		366	222	5·7	0·7	12
		531	**453**	**9·8**	**1·2**	**23**
12–14 Mar.	Casein	**914**	**779**	**10·6**	**2·2**	**52**

Table III. *Puppy N. Weight 1·9 kg.*

Date	Diet	Total N mg.	Creatinine N mg.	Uric acid N mg.	Allantoin N mg.
4 April	Casein	804	8·1	2·1	48
5 „		1003	10·8	3·0	50
6 „		998	9·5	2·2	57
7 „		821	7·8	1·6	70
8 „		843	8·1	1·2	54
9 „		838	7·9	1·4	63
		884	**8·7**	**1·9**	**57**
12 April	Haemoglobin	1119	8·6	2·5	106
13 „		802	7·8	3·9	113
14 „		946	9·7	3·3	109
15 „		1243	6·2	6·7	123
16 „		1072	14·1	4·5	119
17 „		1128	11·8	5·3	121
18 „		1169	10·1	6·3	141
19 „		1012	11·4	4·8	114
		1061	**10·0**	**4·7**	**119**
21–27 April	Casein	**903**	**9·4**	**0·9**	**58**
1 May	Arachin	710	12·2	0·2	46
2 „		553	9·5	0·0	22
3 „		694	—	0·3	38
4 „		454	4·7	0·4	36
5 „		612	6·1	0·1	30
6 „		606	6·1	0·1	32
7 „		498	5·7	0·1	40
		590	**7·4**	**0·1**	**35**
11–16 May	Casein	**918**	**11·5**	**1·0**	**58**

Table IV. *Puppy P. Weight 1·1 kg.*

Date	Diet	Total N mg.	Creatinine N mg.	Uric acid N mg.	Allantoin N mg.
24 Jan.	Casein	811	3·5	1·4	31
25 ,,		789	2·9	1·3	30
26 ,,		684	3·0	1·1	29
27 ,,		674	2·8	0·7	31
28 ,,		693	2·3	0·8	23
29 ,,		654	3·0	1·1	26
30 ,,		663	3·7	1·2	39
		710	**3·0**	**1·1**	**30**
31 Jan.	Haemoglobin	802	3·5	0·7	32
1 Feb.		994	3·0	1·1	33
2 ,,		935	3·1	1·6	32
3 ,,		945	3·0	1·7	43
4 ,,		923	4·0	1·5	46
5 ,,		998	4·1	1·8	49
		933	**3·5**	**1·4**	**39**
6–12 Feb.	Casein	**782**	**3·7**	**1·0**	**45**
13–24 Feb.	Arachin	**434**	**3·8**	**1·0**	**37**
25 Feb.	Arachin (+50%)	535	4·2	0·7	31
26 ,,		556	3·8	0·6	28
27 ,,		579	4·1	0·4	26
28 ,,		624	4·1	0·5	27
		574	**4·1**	**0·6**	**28**

with casein and many days are required to lower the allantoin to a constant, minimal level. The true level for allantoin from haemoglobin was probably not reached and the average figure is misleading.

The variation in the excretion of the total N on the different diets in all three animals is disturbing. The output was highest with haemoglobin and lowest with peanut meal. This may be explained either by the degree of digestion or of utilization of the particular protein. The proportion of protein used by us in the diets is relatively high and contributes about 33% of the total calories. The experimental work on the proteins of the peanut tends to show a digestibility of 96% and a biological value of 56 by the N balance method on the rat [Mitchell *et al.* 1936].

Casein has been assigned a biological value of 74 [Mason & Palmer, 1935]. Jones & Waterman [1922] have found arachin to be somewhat less digestible (48%) than casein (61%) *in vitro*. There are apparently no similar determinations with haemoglobin. In terms of growth response however Baernstein [1938] has recently reported the equality of casein and defatted peanut for the white rat when fed at a level of 20%. The applicability of these facts to the dog under our dietary conditions is questionable. We are inclined to interpret the variations of total N as due to differences in digestibility and absorption. For this reason the last period in Table IV is one with the peanut protein increased by 50% above the previous level. This caused a definite increase in total N but a decrease in purines excreted.

If the excretion of purine N be expressed on a basis of mg. per g. total urinary N the results in Table III would give for casein, 67, for haemoglobin, 112 and for arachin, 59. By this calculation the other two experiments become more uniform. Rose [1921, 2] has shown that the level of protein intake has a comparatively small influence on the excretion of purines in man.

A possible difference in the specific dynamic actions of these proteins might explain the variation in purine excretion. The constancy in the figures for creatinine makes this unlikely however.

We therefore submit these experiments as evidence that the excretion of uric acid and allantoin in the puppy is dependent upon the type of protein in essentially purine-free diets containing a single protein. Haemoglobin, high in histidine, causes the largest excretion and arachin, high in arginine, the lowest.

It is suggested that the conversion of histidine into purines by the puppy occurs in excess of anabolic needs and is demonstrable by increased urinary excretion of uric acid and allantoin, dependent upon the type of dietary protein.

Summary

From feeding experiments with puppies on synthetic diets containing a single protein it has been found that the level of excretion of uric acid and allantoin varies with the protein. Haemoglobin, rich in histidine, causes the largest excretion and arachin, rich in arginine, the lowest, with casein intermediate.

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CXLIX. ON THE PURINE METABOLISM OF THE DALMATIAN COACH HOUND

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The remarkable differences in purine metabolism as to synthesis and uricolysis amongst vertebrates have recently been emphasized by Brunel [1937] in contrasting the mammal with the amphibian, reptile and bird. Even in the mammal the uricolytic index in man and the higher apes is 0–2 whereas in most mammals it is 90–98. In a few it is intermediate as in the horse, 88, sheep, 80 and opossum, 79 [Hunter & Givens, 1914]. This is also true of the New World monkeys [Rheinberger, 1936]. Benedict [1916] first reported the unusual purine metabolism of the Dalmatian coach hound with an index of about 30 and demonstrated the synthesis of purines in this animal on diets essentially purine-free.

The work of Terroine *et al.* [1931–32] with the pig suggests an inverse relationship between the N retention and the level of purine excretion on purine-free diets, high and low in protein. In complete inanition in the rat, as well as on a low protein intake, the loss of tissue purines did not account for the amount excreted. These authors conclude that a synthesis of purines takes place at the expense of either tissue protein or, under normal conditions, dietary protein. Degan [1933] and Kapeller-Adler *et al.* [1934] have come to essentially the same conclusions.

The material for this synthesis in mammals is not established and forms the main subject of this investigation. The literature has been reviewed in the preceding paper [Crandall & Young, 1938] in which evidence supporting the thesis that histidine is the essential amino-acid was obtained from feeding experiments with ordinary puppies on synthetic diets containing casein, haemoglobin or arachin.

The Dalmatian provides an experimental animal excreting considerable amounts of uric acid and therefore suited to metabolic experiments with synthetic diets selected to contrast the effects of different amino-acids on the excretion of purines.

Experimental

Two Dalmatian bitches, 3 months old, of registered pedigrees, were confined in metabolic cages intermittently over a period of several months. The urine, collected under toluene after passing through a fine mesh screen, was removed every 24 hr. The daily output of about 350 ml. was diluted to 1 litre and the reaction adjusted to neutrality or slight alkalinity. On the diets given the urine tended to be alkaline and to deposit crystalline ammonium magnesium phosphate. The dogs were weighed every 4 or 5 days. For the greater part of the experiment they gained slowly in weight. At no time were the dogs ill, but they lost their appetites on certain diets. Water was given *ad libitum* and food twice daily.

Methods. Urinary constituents were determined by the following methods: total N, Kjeldahl; urea and NH_3, Van Slyke-Cullen; creatine and creatinine, Folin micro-methods; uric acid, Christman & Ravwitch [1932]; allantoin, Larson [1932] and occasionally Christman [1926].

Diets. In general we have followed the basal requirements of Cowgill [1923]. Previous experience with puppies led us to adopt the levels of 100 Cal. and 1 g. protein N per kg. body wt. To the calculated amount of protein, previously analysed, were added sucrose and lard such that the fat did not exceed 25 % of the total weight of the daily ration. Yeast concentrate, halibut liver oil, salt mixture and bone ash were added as previously described [Crandall & Young, 1938]. Single proteins were fed on the basis of N equivalence, forming the only variable in the diet. The period for each diet was approximately 1 week but depended on palatability and the constancy in the level of excretory products. Casein was adopted as standard protein for comparison, and other proteins selected to provide a high intake of particular amino-acids were arachin (arginine), haemoglobin (histidine), serum albumin (cystine), gelatin (glycine). Serum albumin and haemoglobin are also high in leucine and *iso*leucine, casein in glutamic and hydroxyglutamic acids, and gelatin in proline and hydroxyproline.

Haemoglobin and a mixture of arachin with conarachin were prepared as in the preceding paper [Crandall & Young, 1938]. The casein, serum albumin and gelatin were commercial samples in powdered form. The meat protein was obtained from minced lean round steak by boiling in water at *p*H 5 for 15 min., filtering, re-mincing and re-boiling twice, and drying in air. All protein preparations used were analysed for their content of moisture, protein, fat and ash. These figures were used in the calculations of the specific diets recorded in Tables I and III.

There is a disturbing discrepancy in the values for histidine in casein recorded in the literature. The most recent figure of 11 % [Kapeller-Adler, 1933] is almost double the older values of 6·2–6·6 % of Van Slyke and Crowther & Raistrick, and if confirmed this value would disturb the contrast between casein and haemoglobin.

Preformed purines in diets. These were determined in our protein preparations after initial hydrolysis by the method of Graff & Maculla [1935] and the results were as follows: casein, 14; haemoglobin, 43; peanut meal, 17; serum albumin, 22; gelatin, 0; meat powder, 35; purina powder, 24; expressed as mg. purine N per 100 g. stock preparation. The amounts of preformed purines consumed daily are listed in Tables I and III. These values are not insignificant but are small compared with the fluctuations observed and in relation to the total purine excretion. The dietary purine N would be about 5–10 % or less of the urinary purine N in Exp. I and 10 % or less in Exp. II.

Exp. I. Tanya of Tattoo

The sequence of the diets and their composition are given in Table I, including the calculated consumption of particular amino-acids for comparison with the amounts of uric acid and allantoin excreted. The calculations have been made for the most part from the data given by Mitchell & Hamilton [1929]. Where any discrepancies in sequence of dates occur the animal had been removed from the cage and fed the diet of the subsequent period.

The first three diets were given at a level of 100 Cal. and 1 g. protein N per kg. body wt. Because the weight of the animal remained stationary during this period the level was increased to 150 Cal. and 1·5 g. protein N for the diets following, introduced by a long period of 3 weeks on casein. A period of 6 days without food followed to determine minimal levels of purine excretion after the

Table I. *Composition of diets*

Diet	Wt. of animal kg.	Protein N per kg. g.	Cal. per kg.	Protein g.	Lard g.	Sucrose g.	Purine N mg.	Arginine N g.	Histidine N g.	Lysine N g.	Cystine N g.	Glycine N g.
Casein	4·7	1·0	100	39	20	40	5·5	0·348	0·292	0·485	0·094	0·023
Arachin	4·9	1·0	100	44	10	46	6·8	1·163	0·137	0·254	0·034	0·103
Haemoglobin	5·0	1·0	100	32	20	48	13·7	0·384	0·635	0·545	0·000	0·023
Casein	5·1	1·5	150	63	32	64	8·4	0·563	0·472	0·784	0·015	0·037
Serum albumin	7·5	1·5	150	94	48	75	20·8	1·175	0·757	1·840	0·395	0·000
Gelatin	6·9	1·5	150	73	40	90	0·0	1·513	0·463	0·650	0·010	2·740
Casein (minimum)	6·8	0·35	74	20	20	65	2·8	0·178	0·149	0·248	0·005	0·012
Casein	7·3	1·0	100	60	20	88	8·4	0·539	0·453	0·753	0·015	0·035

unsuccessful use of placental protein. Gelatin was next given because of its incompleteness and to stimulate endogenous metabolism by the specific dynamic action of glycine. This was followed by casein given first at a minimum level corresponding with the endogenous requirement of the inanition period and then on the initial basis. The dog lost weight only during the starvation period.

Table II. *Average composition of urine*

Date	Diet	Diet N g.	Total N g.	Creatinine N mg.	Creatine N mg.	Uric acid N mg.	Allantoin N mg.	Uricolytic index
8–12 April	Casein	4·7	2·16	31·6	43·2	119	228	66
13 April–10 May	Arachin	4·9	2·15	33·4	47·8	59	228	80
15–22 May	Haemoglobin	5·0	4·31	34·8	32·2	38	220	85
24 May–13 June	Casein	7·6	4·08	43·9	15·6	72	318	82
18–29 June	Serum albumin	11·3	4·71	56·0	9·1	88	308	78
3–8 July	No food	—	2·44	67·9	14·5	66	126	66
9–10 July	Gelatin	10·3	6·76	61·9	30·9	62	567	90
17–23 July	Casein	2·4	1·84	61·5	21·4	65	249	79
25–30 July	Casein	7·3	3·76	70·1	9·8	71	347	83

The results of the urinary analyses are tabulated as averages for the respective periods in Table II. During the first three periods the only notable change was a decline in the level of uric acid excretion. The allantoin remained remarkably constant despite the fact that in the peanut meal three times more arginine was ingested and one-fifth the amount of histidine. Although the values for uric acid in the introductory period were constant we do not consider the level as representative of the casein but rather of the previous dietary history. This is evident especially when compared with the second longer casein period with a higher intake of protein and a higher total N yet a lower uric acid output. In this period it required over 1 week for the animal to reach the maximum level of excretion of total N. The serum albumin provided the highest intake of cystine and also of histidine and lysine yet the products of excretion were essentially the same as for the previous casein period. At this high level of protein intake there is a marked difference between the total N of the food and of the urine.

Inanition for 6 days reduced the excretion of uric acid to the level observed on previous diets and the allantoin to the lowest recorded. Gelatin for 2 days at this juncture had a tremendous effect on the excretion of allantoin but did not affect the uric acid, due possibly to active synthesis of tissue proteins after starvation, or the conversion of protein into carbohydrate, or the appearance of some urinary compound other than allantoin affecting the Larson copper reduction method. The final periods at different levels of casein intake demonstrate the relative independence of uric acid but the response of allantoin.

Generalizing from this experiment uric acid excretion has been found to be remarkably independent of diet. Allantoin on the other hand fluctuates without convincing relationship to any amino-acid studied and follows more closely the total N of the urine.

Exp. II. Topper of Tattoo

The sequence and composition of diets with this animal are recorded in Table III. We began this experiment by contrasting (with the adopted normal) the effects of diets high and low in casein. With these two exceptions all diets were computed on the basis of 100 Cal. and 1 g. protein N per kg. body wt.

Table III. *Composition of diets*

Diet	Wt. of animal kg.	Protein N per kg. g.	Protein g.	Lard g.	Sucrose g.	Purine N mg.	Arginine N g.	Histidine N g.	Lysine N g.	Cystine N g.	Glycine N g.
Casein-normal	9·3	1	68	25	117	9·5	0·69	0·58	0·96	0·020	0·045
Casein-minimum	9·5	0·3	26	39	126	3·6	0·24	0·20	0·33	0·006	0·015
Casein-maximum	9·5	2	158	30	6	22·2	1·41	1·18	1·96	0·038	0·091
Serum albumin	10·0	1	90	60	36	19·8	1·04	0·67	1·63	0·443	0·000
Arachin	9·6	1	117	40	62	19·9	2·28	0·27	0·50	0·067	0·202
Haemoglobin	10·4	1	70	50	88	30·2	0·80	1·32	1·13	0·000	0·046
Gelatin	10·3	1	68	50	87	0·0	1·52	0·46	0·65	0·010	2·740
Casein	10·1	1	89	50	89	12·5	0·75	0·63	1·04	0·020	0·485
Meat proteins	11·0	1	83	60	65	29·0	1·46	0·55	1·23	0·121	0·258

Table IV. *Average composition of urine*

Date	Diet	Diet N g.	Total N g.	Creatinine N mg.	Creatine N mg.	Uric acid N mg.	Allantoin N mg.	Uricolytic index
14–27 Jan.	Casein-normal	9·3	6·81	79	17	88	88	50
28 Jan.–4 Feb.	Casein-minimum	3·2	2·43	79	19	82	101	55
5–7 Feb.	Casein-maximum	19·0	16·34	93	14	126	129	51
16–23 Feb.	Serum albumin	10·0	9·47	88	4	100	40 (?)	29
27 Feb.–8 Mar.	Arachin	9·8	6·41	80	2	85	150	64
9–13 Mar.	Haemoglobin	10·4	8·96	100	0	112	110	50
17–23 Mar.	Gelatin	10·3	10·84	100	63	57	55	49
24 Mar.–1 April	Casein	10·1	6·77	89	16	110	?	?
22–30 April	Meat protein	11·0	5·74	102	47	61	115	65

The salient points observable from a comparison of Tables III and IV may be summarized as follows.

(1) A reduction in the protein N intake from 9·3 to 3·2 g. made no difference in the excretion of uric acid and allantoin. An increase to 19·0 g. with a very high urinary N augmented the level of purine excretion very slightly, but in our opinion significantly.

(2) The uricolytic index was characteristic of the diet as well as of the dog. Thus casein, haemoglobin and gelatin provided an index of 50 while serum albumin lowered it to 29 and peanut meal raised it to 65. The figures for allantoin obtained with serum albumin and in the last casein period are in some doubt and are probably too low, due to a tendency for cloudiness to develop during the determination. In the last casein period the determinations obtained were too few to warrant an average.

(3) The exogenous supply of cystine cannot be related to the purine excretion. On the minimum casein diet the intake of cystine N was 6 mg. and the output of purine N was 184 mg. On the serum albumin diet the intake was 443 mg. and the output 140 mg.

(4) The effect of gelatin is noteworthy in that despite a negative N balance, the excretion of total purines is the lowest in the experiment. The effect on the excretion of creatine is interesting and was also observed in the first animal (Table II). It suggests a sudden synthesis from glycine.

(5) The relationship of either arginine or histidine to purine synthesis is not obvious. On the second lowest intake of histidine, 270 mg., in peanut meal, we obtained the highest excretion of allantoin. Arginine appears more closely related to purine excretion but this relationship is not demonstrated in convincing manner in this experiment. Thus on the minimum casein diet containing 240 mg. arginine N, 184 mg. purine N were excreted. On the maximum arginine content in peanut meal however we did obtain the highest excretion of allantoin, despite a low figure for the total urinary N. This points to a possible further difference between the Dalmatian and other dogs in their purine metabolism.

Effect of supplementary amino-acids

Following the usual procedure of adding pure amino-acids to a basal diet in an effort to demonstrate a linear relationship to an excretory product we have used arginine, histidine and glycine. At this time the dog tended to lose her appetite and would not consume the daily rations quantitatively. We were compelled to make use of coagulated protein from ox muscle as basal diet. Material, prepared identically, has been analysed by Rosedale [1922] and Jones *et al.* [1924] whose analyses have been used in Table III: this powder however contained some nucleoprotein. After an introductory period the amino-acids, as hydrochlorides, were added. The Christman method for allantoin was used in these analyses. The variation of purine excretion on the basal diet is somewhat disconcerting-especially with a constant excretion of creatinine. The output of uric acid rose slightly after both arginine and histidine, and that of allantoin definitely after 3 g. histidine. The histidine effect was repeated later with the same animal and compared with an equivalent amount of glycine but the latter was without effect.

Effect of preformed purines

Our results on the administration of preformed purines, either free or in combined form as nucleic acid, have tended to show a constancy in uric acid as compared with allantoin. We wished to know whether the present conceptions of pathways to uric acid and allantoin held in the Dalmatian. Wells [1918] has shown the existence in the liver of adenase, guanase and uricase but the absence of xanthine oxidase. The effect of feeding thymus was an increased elimination of both uric acid and allantoin with a rise in the uricolytic index as observed by Benedict [1916]. Allen & Cerecedo [1933] have found that guanine is metabolized to allantoin and in part to urea in the ordinary dog.

The purines used in our experiments were obtained from Hoffmann-La Roche Inc., Basel, Switzerland. We have added them in capsules to a basal diet of purina dog chow. This became necessary because the dog could not be depended upon to maintain her appetite for a sufficiently long period otherwise. The ordinary checkers were ground, moistened and used as the basal ration unsupplemented. In that the daily portion was consumed quantitatively the presence of preformed purine material was not considered a serious objection.

The Larson method for allantoin was again used in this series of experiments.

Table V represents the results condensed where possible. Guanine and xanthine caused appreciable increases in allantoin. Uric acid itself, *per os* in small amounts, had a similar effect. Nucleic acid increased the output of uric acid slightly, allantoin and urea definitely. At times notable increases in NH_3

Table V. *Effects of supplementary purines*

Date	Total N g.	Urea N g.	Ammonia N g.	Creatine N mg.	Creatinine N mg.	Uric acid N mg.	Allantoin N mg.	Uricolytic index	Remarks
29 May–2 June	7·04	5·54	0·732	40	105	125	310	71	Basal diet
3 June	7·69	6·33	0·687	43	99	140	352	72	+1 g. guanine
4 ,,	6·95	5·64	0·626	48	99	116	452	80	
5 ,,	8·78	6·78	0·914	57	99	148	532	78	
	7·81	6·25	0·742	49	99	135	445	77	
6 June	6·89	5·38	1·237	14	88	106	400	79	+0·5 g. xanthine
7 ,,	8·33	7·11	0·847	60	92	141	395	74	+0·5 g. xanthine
8 ,,	9·95	8·23	0·810	14	103	121	585	83	
9 ,,	7·89	6·36	0·634	14	103	103	490	83	
10 ,,	8·16	6·92	0·902	48	99	111	479	81	
	8·24	6·80	0·798	30	97	116	470	80	
11 June	7·41	5·84	0·677	37	95	87	300	77	+0·5 g. uric acid
12 ,,	8·13	6·50	0·558	43	99	119	346	75	+0·5 g. uric acid
13 ,,	8·39	6·45	1·167	17	95	89	441	83	
14 ,,	7·75	6·53	0·646	39	99	121	350	74	
15 ,,	8·74	7·24	0·714	42	115	112	425	79	
	8·08	6·51	0·752	36	101	106	372	78	
16 June	7·58	6·12	0·573	40	100	82	350	81	+1 g. nucleic acid
17 ,,	8·95	7·13	0·733	40	101	136	690	83	+2 g. nucleic acid
18 ,,	9·77	8·04	0·774	32	110	129	438	77	+2 g. nucleic acid
	8·77	7·10	0·693	37	104	116	493	81	
19–28 June	7·74	6·42	0·757	42	99	91	377	80	

are recorded. They may represent bacterial fermentation in the urine during collection or a degradation of the purine nucleus. The most remarkable observation in this experiment was the constancy in the excretion of uric acid despite distinct changes in the excretion of allantoin. It suggests that in the Dalmatian there is an endogenous metabolism of uric acid distinct from that of allantoin.

A difference has been noted in results of administering purines by capsule or mixed with the food. In the latter method adenine and guanine appear to be degraded and excreted in a non-purine form. We have fed hypoxanthine and xanthine in quantities of 1 g. in like manner but without any perceptible effect on the N excretory products.

Discussion

Briefly stated there are three conceptions of the origin of urinary purines in mammals on essentially purine-free diets. The first empirically relates the production of purines to the extent of the metabolism of carbohydrates. The second draws a parallel between the specific dynamic action of food and the catabolic production of purines. This indicates a closer but non-specific relationship to endogenous metabolism. The third pictures a synthesis from a nitrogenous precursor to provide nuclear material for growth and repair with excretion of plethora as uric acid or allantoin. This indicates a specific relationship to exogenous metabolism. The experiments in this paper have been based on the third hypothesis. The behaviour of allantoin in our experiments appears to fit the third conception and that of uric acid the second. But the evidence presented above does not indicate any apparent specificity and on the same diet there was considerable fluctuation from day to day. The daily volume of urine was not measured and may have accounted for this variation as has been suggested by Krafka [1932].

The uricolytic index of the Dalmatian as recorded by different observers varies considerably. Our first dog, Tanya, showed an index of 66–90, average, 80;

our second dog, Topper, 29–65, average 50. The first dog studied by Benedict had a variation of 24–34 on a low protein diet and 42–47 on a high, as calculated by us from his data. In another experiment this dog showed an index of 62. The adult female studied by Onslow [1923] exhibited a variation of 72–93, average 84, on a mixed diet. Such variation is comprehensible if uric acid is essentially endogenous, and allantoin excretion varies with the diet and other physiological conditions.

In view of the relative constancy in the excretion of uric acid we have calculated the output on the basis of mg. per kg. body wt. A comparison of our figures with those of others, in so far as it was possible to calculate these, is given below. The figures given for man were calculated from the data of Folin [1905] on diets low and high in protein and essentially free from preformed purines.

Animal	Reference	Excretion of uric acid mg./kg.
Dalmatian	Authors	35
,,	,,	30
,,	Benedict [1916]	36
,,	Wells [1918]	37
,,	Givens [quoted by Wells, 1918]	40
,,	,, ,,	64
,,	Watanabe [1917]	12
,,	,,	3
,,	Onslow [1923]	4
Ordinary dog	Crandall & Young [1938]	4
Man	Folin [1905]	5–8

It is thus apparent that the true Dalmatian excretes uric acid to an approximately constant extent of 30–40 mg./kg., and that this is of greater constancy than the uricolytic index. It suggests that the dogs used by Onslow and Watanabe were not typical Dalmatians as regards their purine metabolism. It supports the statement that per kg. body wt. the Dalmatian excretes much more uric acid than man.

Summary

Two Dalmatian puppies have been subjected to metabolic experiments on synthetic diets containing single proteins to contrast the excretion of uric acid and allantoin with the dietary content of particular amino-acids. Casein has been used as basal protein.

The amounts of dietary protein required to supply the precursor in purine synthesis is less than 0·3 g. N per kg. body wt. The uricolytic index is characteristic of the diet and of the individual dog, varying from 29 to 90. The evidence for a particular precursor in the synthesis of purines is not conclusive. Cystine is definitely eliminated. Neither histidine nor arginine shows a clear relationship. Using amino-acids supplementary to a basal diet both histidine and arginine cause a slight increase in the output of allantoin but not of uric acid.

Guanine, xanthine, uric and nucleic acids administered by capsule increased the output of allantoin and of urea and raised the uricolytic index.

The excretion of uric acid has been so notably constant throughout the experiment as to suggest it being essentially an endogenous metabolite. It was found to be 30–40 mg. per kg. body wt., and this criterion was more characteristic of the Dalmatian than the uricolytic index.

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CL. GLYOXALINES: THE DETERMINATION OF THEIR *p*K VALUES AND THE USE OF THEIR SALTS AS BUFFERS

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The work described in this paper was undertaken for two reasons: first, there is a lack of buffers for use between *p*H 6 and 9 in experiments where phosphates are excluded. Further, it is generally desirable, particularly in physico-chemical investigations, to use buffers of a symmetrical valency type; these should preferably be uni-univalent salts.

The *p*H of buffer solutions is usually determined by the electrometric technique involving cells with liquid junctions, although it is now recognized that measurements with such cells do not generally yield accurate values for either the concentration or the mean activity of H^+ ions. The magnitude which we call *p*H is really a function which depends on the concentrations, valencies and mobilities of all the ions present in the solution and is generally not an accurate measure of the acidity. Only in cases where the total ionic concentration is low and the different ions are of low valency type, can the Debye-Hückel approximations be applied. If in such cases precautions are taken to make the liquid junction potential small, the *p*H values obtained represent a fairly good approximation of the mean activity of H^+ ions which is the true measure of the acidity of the solution.

In physico-chemical work with compounds of high valency such as proteins the advantage of using uni-univalent buffers is even more obvious. The Debye-Hückel formulae, which already fail with concentrated solutions of uni-univalent salts, break down for dilute solutions of salts of higher and unsymmetrical valency type [La Mer *et al.* 1927]. It is certainly not to be expected that the Debye-Hückel formulae and the principle of ionic strength should apply to protein solutions [*v.* Hartley, 1935]. Ions of higher valency interact with oppositely charged protein ions much more strongly than can be accounted for on the basis of Debye-Hückel formulae, a phenomenon which is predictable from Bjerrum's theory of ionic association: such a type of Coulomb interaction affords an explanation of the experiments of Tiselius [1930; 1937], who found that the electric mobilities of protein ions at constant *p*H were altered by substituting multivalent for univalent buffers. Shifts in the isoelectric points of proteins produced by salts of different valency type [Smith, 1935; 1936] and the differences between "isoionic" and "isoelectric" points as in the experiments of Adair & Adair [1934] can also be explained by the fact that in protein systems the principle of ionic strength breaks down and has to be replaced by theories of the type suggested by Bjerrum [1926] and Hartley [1935]. In all these cases the difficulties of even qualitative interpretation are much enhanced by the use of buffers of unsymmetrical valency type. The use of uni-univalent buffers will

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not eliminate all difficulties, since specific properties of ions may come into play in colloidal systems, but it is reasonable to expect that the interpretation of measurements of this character will be greatly facilitated.

For substances to be suitable as the basis of a system of buffers of the type desired certain criteria must be fulfilled. The required range can scarcely be covered with less than three compounds which should have *p*K approximately 6, 7 and 8; the compounds should be stable and easily accessible and, since the buffer solutions are likely to be employed in biological experiments, they should if possible be biologically indifferent.

A search of the literature indicated that these requirements were probably most nearly satisfied by bases of the glyoxaline series; glyoxaline itself has *p*K in the neighbourhood of 7 and it seemed likely, particularly in view of the few data concerning the dissociation constants of substituted glyoxalines which are already recorded in the literature, that the introduction of suitable electron-attractive or electron-repellent groups into the molecule would yield derivatives having the required *p*K of about 6 and 8. As will be seen below this expectation has been fulfilled.

So far as stability is concerned the glyoxalines leave little to be desired; buffer solutions prepared from them have remained unchanged over 3 weeks without any elaborate precautions and there is no reason to suppose that their actual period of stability is not much longer. A large variety of substituted glyoxalines have recently become readily accessible through the simple synthetical method of Weidenhagen & Herrmann [1935]. Finally, so far as is at present known, the glyoxalines used in the present work have no such specific effects on enzyme systems as have been found for instance to be troublesome in the case of the veronal buffers of Michaelis [Maeda, 1937].

Experimental

Preparation of compounds

Glyoxaline was prepared from nitrotartaric acid according to the method of Fargher & Pyman [1919], being purified by distillation and finally by recrystallization from benzene. 4 (or 5)-Methylglyoxaline was prepared from glucose by the method of Windaus & Knoop [1905] and purified by distillation followed by conversion into the picrate; the latter was decomposed with HCl and benzene and the aqueous solution evaporated; the residual hydrochloride was crystallized from absolute alcohol. The hydrochloride, which is hygroscopic, has M.P. 118°. (Found: N (Kjeldahl) 23·7; Cl, 30·6 %. $C_4H_2N_2Cl$ requires N, 23·6; Cl, 30·0 %.)

4(or 5)-Hydroxymethylglyoxaline was prepared according to Weidenhagen & Herrmann [1935] from dihydroxyacetone (oxantin); it was purified through the picrate and finally converted into the hydrochloride, which had M.P. 110° (values given in the literature range from 93 to 109°).

2:4(or 5)-Dimethylglyoxaline was obtained by the method of Weidenhagen & Herrmann [1935] except that acetol (*Org. Syntheses*, **10**, 1) was used instead of acetol acetate. This base was either purified by distillation or converted via the picrate into the hydrochloride, which, after recrystallization from alcohol, had M.P. 207° (uncorr.). (Found: N, 21·2; Cl, 27·0 %. $C_5H_9N_2Cl$ requires N, 21·2; Cl, 26·8 %.)

All the other glyoxaline derivatives were prepared exactly according to Weidenhagen & Herrmann [1935], and had M.P. in agreement with the values recorded in the literature.

Table I

Substance	$E_{corr.}$	pH	α	μ	pK_0
Glyoxaline	0·7533	7·074	50·0	0·1050	6·95
	0·7525	7·062	50·0	0·0750	6·96
	0·7513	7·041	50·0	0·0450	6·95
	0·7506	7·028	50·0	0·0250	6·96
	0·7496	7·011	50·0	0·0150	6·96
	0·7490	7·001	50·0	0·0090	6·96
	0·7221	6·546	75·0	0·0475	6·93
	0·7790	7·509	25·0	0·0425	6·95
2-Methylglyoxaline	0·8066	7·977	50·0	0·1050	7·86
	0·8057	7·960	50·0	0·0750	7·85
	0·8049	7·947	50·0	0·0450	7·86
	0·8037	7·927	50·0	0·0250	7·86
	0·8032	7·918	50·0	0·0150	7·86
	0·8024	7·905	50·0	0·0090	7·86
	0·7746	7·435	75·0	0·0475	7·82
	0·8322	8·410	25·0	0·0425	7·85
4(or 5)-Methylglyoxaline	0·7871	7·645	50·0	0·1050	7·52
	0·7857	7·623	50·0	0·0750	7·52
	0·7841	7·595	50·0	0·0450	7·51
	0·7832	7·580	50·0	0·0250	7·51
	0·7830	7·577	50·0	0·0150	7·52
	0·7830	7·577	50·0	0·0090	7·53
	0·7556	7·112	75·0	0·0475	7·50
	0·8133	8·089	25·0	0·0425	7·53
2:4(or 5)-Dimethylglyoxaline	0·8366	8·482	50·0	0·1050	8·36
	0·8355	8·465	50·0	0·0750	8·36
	0·8345	8·448	50·0	0·0450	8·36
	0·8334	8·429	50·0	0·0250	8·36
	0·8327	8·418	50·0	0·0150	8·36
	0·8322	8·410	50·0	0·0090	8·37
	0·8057	7·960	75·0	0·0475	8·35
	0·8618	8·910	25·0	0·0425	8·35
4(or 5)-Hydroxymethylglyoxaline	0·7200	6·510	50·0	0·1050	6·39
	0·7184	6·483	50·0	0·0750	6·38
	0·7174	6·468	50·0	0·0450	6·38
	0·7166	6·454	50·0	0·0250	6·39
	0·7160	6·443	50·0	0·0150	6·39
	0·7160	6·443	50·0	0·0090	6·40
	0·6887	5·981	75·0	0·0475	6·37
	0·7454	6·940	25·0	0·0425	6·38
2-Phenylglyoxaline	0·7177	6·473	51·0	0·0550	6·40
	0·7164	6·449	51·0	0·0350	6·39
	(0·7166)	—	51·0	0·0250	—
	0·7157	6·439	51·0	0·0190	6·40
	0·7161	6·445	51·0	0·0150	6·41
	0·6881	5·971	76·5	0·0375	6·40
	0·7460	6·950	25·5	0·0325	6·41
4(or 5)-Phenylglyoxaline	0·6973	6·127	50·0	0·1050	6·01
	0·6949	6·087	50·0	0·0750	5·98
	0·6942	6·074	50·0	0·0450	5·99
	0·6940	6·071	50·0	0·0250	6·00
	0·6933	6·059	50·0	0·0150	6·00
	0·6653	5·586	75·0	0·0475	5·97
	0·7254	6·603	25·0	0·0425	6·04

Table II

Substance	Mean pK_0 value
Glyoxaline	6·95
2-Methylglyoxaline	7·86
4(or 5)-Methylglyoxaline	7·52
2:4(or 5)-Dimethylglyoxaline	8·36
4(or 5)-Hydroxymethylglyoxaline	6·38
2-Phenylglyoxaline	6·39
4(or 5)-Phenylglyoxaline	6·00

Measurements of the dissociation constants

The dissociation constants were measured in cells of the type:

Pt, H_2	0·01 *M* base *X M* HCl *Y M* KCl	3·5 *M* KCl	0·1 *M* KCl HgCl	Hg

where $X < 0{\cdot}01$ and Y varied between 0·1 and 0·004 *M*. Except in the most dilute solutions the concentrations of K^+ and Cl^- were much larger than the sum of all the other ions. The liquid junctions were made inside a tube of internal diameter 3 mm. and were very sharp initially. The potentiometer was a Tinsley instrument Type 4025; the galvanometer was of the D'Arsonval type with an internal resistance of 400 ohms. All the measurements were made at 25° ± 0·05.

The main difficulty in measurements with cells containing liquid junctions is that errors due to liquid junction potentials introduce uncertainty about the correct value of E_0. The liquid junction potentials in systems of the type described above are certainly not greater than 0·5 mV. Both half-cells contain K^+ and Cl^- ions and are nearly symmetrical and the two liquid junctions should cancel each other to a very large extent. Variations in the KCl concentration in the one half-cell will cause definite changes in the liquid junction potential, but these may be neglected as shown by the fact that extrapolation of the apparent *p*K values to ionic strength leads to fairly constant pK_0 values. Liquid junction potentials have been in fact neglected throughout this work.

The values for E_0 given in the literature vary considerably. Guggenheim & Schindler [1934] chose a value of 333·7 mV., whilst in an earlier paper by one of us [Neuberger, 1937] a value of 334·4 mV. was used. A few experiments on the acetic acid-sodium acetate system showed that the best average *p*K value of acetic acid was obtained when E_0 was taken as 335·1 mV., if the liquid junction potential is neglected. Taking into account the slight remaining uncertainty which cannot be completely eliminated it is assumed that the *p*K values will be correct within 0·01.

The results obtained with the different compounds are recorded in Table I. E is corrected for barometric pressure, α is the degree of neutralization of the base with acid and μ is the ionic strength. pK_0 is calculated from the apparent *p*K by the formula

$$pK_0 = pK - \frac{0{\cdot}5\sqrt{\mu}}{1+\sqrt{\mu}}.$$

Table II contains the mean values for pK_0 obtained from the data of Table I.

Discussion

The introduction of the electron-repelling methyl group increases the basicity very considerably. The effect of substitution in position 2 is much stronger than the effect of substitution in position 4 (or 5), whilst substitution of two methyl groups in positions 2 and 4 (or 5) increases the *p*K values even more strongly. The difference in *p*K values between glyoxaline and the dimethyl derivative is nearly equal to the sum of the differences between glyoxaline and the 2 and the 4 (or 5) monomethyl derivatives respectively, indicating that the effect of substitution is additive.

The electron-attracting phenyl group produces the opposite effect, but here substitution in position 4 (or 5) causes a smaller change in *p*K than substitution in position 2, an observation which is difficult to interpret. The diphenyl derivative is too insoluble in water to allow any accurate measurements to be made by the technique used in this work.

As might be expected from its electronegative character the hydroxymethyl group causes a decrease of pK. Generally it may be noted that the effect of substitution on the pK of glyoxalines is very much larger than the effect of substitution in aliphatic systems or even in the benzene ring. Effects of similar order of magnitude have already been observed, however, in other heterocyclic ring systems, such as pyridine and pyrazole. It seems that electronic displacements in systems containing conjugated double bonds are transmitted to the dissociating centres much more readily than in more saturated compounds.

A further point which makes a comprehensive interpretation of the effect of substitution on pK in glyoxalines more difficult is the existence of tautomerism. The free bases may exist in the forms

In glyoxaline itself and in the bases substituted in position 2 only, the two forms are identical. But for 4 (or 5) monosubstituted bases and for 2:4 (or 5) disubstituted compounds the two forms are different and will have different dissociation constants. The pK values obtained give a measure of the basicity of the equilibrium mixtures only and it is not possible to derive any dissociation constants for the two bases separately. For the corresponding cations it may be assumed that no tautomerism but only resonance is involved, as is the case with guanidine and amidine salts. The charge then will not reside at one particular nitrogen atom but will be distributed over the molecule as a whole.

Substitution would then influence the pK not only by the intrinsic effect of substitution as such, but also by shifting the equilibria between the tautomeric forms of the bases and by the effects caused on the resonance of the cations.

Buffers

The substances which were finally selected as buffers were the hydrochlorides of 4(or 5)-methylglyoxaline, 4(or 5)-hydroxymethylglyoxaline and 2:4(or 5)-dimethylglyoxaline; these compounds are very soluble in water, are fairly easily accessible and together cover the desired pH range satisfactorily. Three series of solutions were made up as shown in Table III; the E.M.F. values were measured at once for each solution and measured again after 3 weeks' storage in the cold room. The potentials found and the pH values calculated from these measurements are recorded in Table III[1] and it can be seen that the buffers have remained unaltered over this period. The buffers have a constant ionic strength; if any other buffer concentration is desired the pH values can be calculated from the formula

$$p\mathrm{H} = p\mathrm{K} - \log \frac{[B.\mathrm{HCl}]}{[\text{Free base}]} + \frac{0{\cdot}5\sqrt{\mu}}{1+\sqrt{\mu}}.$$

Table III shows that the range between pH 5·4 and 9·3 is satisfactorily covered by the new buffers and it is now possible to use uni-univalent buffers in aqueous systems throughout, since the range from pH 3·6 to 5·6 is covered by acetic acid-sodium acetate, whilst glycine-sodium hydroxide mixtures are available for the range 8·6–11·5.

[1] It must be remembered that the figures in Table III are valid for 25° only; the temperature coefficient may be of considerable magnitude.

Table III. *Data for the preparation of buffer solutions*

In each case 25 ml. $N/10$ solution of the base were treated with the number of ml. $N/10$ NaOH shown in col. 2 and the whole made up to 50 ml.

Substance	ml. of $N/10$ NaOH	E observed initially	E observed after 3 weeks	pH observed initially	pH observed after 3 weeks	Mean pH
4(or 5)-Hydroxymethylglyoxaline hydrochloride	2·5	0·6578	0·6581	5·46	5·47	5·47
	5·0	0·6795	0·6808	5·83	5·85	5·84
	7·5	0·6944	0·6955	6·09	6·10	6·10
	10·0	0·7061	0·7070	6·28	6·30	6·29
	12·5	0·7175	0·7188	6·48	6·50	6·49
	15·0	0·7282	0·7291	6·64	6·67	6·66
	17·5	0·7418	0·7419	6·88	6·88	6·88
	20·0	0·7567	0·7568	7·14	7·14	7·14
4 (or 5)-Methylglyoxaline hydrochloride	5·0	0·7499	0·7508	7·02	7·04	7·03
	7·5	0·7631	0·7640	7·24	7·26	7·25
	10·0	0·7751	0·7760	7·45	7·46	7·46
	12·5	0·7861	0·7868	7·64	7·65	7·65
	15·0	0·7960	0·7978	7·80	7·83	7·82
	17·5	0·8077	0·8093	7·98	8·03	8·01
	20·0	0·8221	0·8220	8·24	8·24	8·24
2:4(or 5)-Dimethylglyoxaline hydrochloride	7·5	0·8112	0·8109	8·06	8·05	8·06
	10·0	[0·8178]	0·8225	—	8·25	8·25
	12·5	0·8330	0·8334	8·43	8·44	8·44
	15·0	0·8428	0·8440	8·59	8·62	8·61
	17·5	0·8541	0·8552	8·79	8·80	8·80
	20·0	0·8670	0·8679	9·00	9·03	9·02
	22·5	0·8850	0·8857	9·31	9·32	9·32

SUMMARY

1. It is pointed out that buffers of a symmetrical valency type, especially uni-univalent buffers, give the best-defined pH values; the particular advantage of the use of such buffers in the physico-chemical study of proteins is discussed.

2. The pK values of glyoxaline and some methyl-, phenyl- and hydroxymethyl-substitution products have been measured and are discussed in relation to the structures of the compounds.

3. 4(or 5)-Methyl-, 2:4(or 5)-dimethyl- and 4(or 5)-hydroxymethyl-glyoxaline with hydrochloric acid are proposed as suitable buffers for the range between pH 5·4 and 9·3. The whole pH range from pH 3·6 to 11·5 can thus now be covered with uni-univalent buffer systems.

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CLI. THE LYSINE CONTENT OF FEEDING STUFFS

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Osborne & Mendel [1914] demonstrated conclusively that lysine was an essential amino-acid for normal growth, and drew attention to the importance of studying the qualitative as well as the quantitative aspects of proteins in nutrition. A year later Osborne *et al.* [1915] showed that the nutritive values of a number of proteins were closely related to their lysine contents. More recently attention has again been drawn to the importance of lysine by Morris & Wright [1933]. They state: "...when minimal quantities of protein are fed in the production rations of milking cows, a deficiency of lysine or tryptophan will lead to a marked reduction in milk yield."

The necessity of having some knowledge of the nature of proteins for nutritional studies has led to the publication of a large number of analyses of proteins isolated from feeding stuffs and protein concentrates. Thus Morris [1934] reports the results of analyses of a number of feeding stuffs used in Great Britain, including with his data values obtained by other workers. Almost all of the analyses, however, were made by the Van Slyke [1911] nitrogen distribution method, which has been widely criticized, particularly as a method for determining lysine [cf. Mitchell & Hamilton, 1929; and also Plimmer & Lowndes, 1938]. Recently Block [1934, 1] has shown that dilute alkali and dry heat produce some change in protein structure which is indicated by an abnormal and high value for lysine when determined by this method, so that most of the above-mentioned results may be further criticized since the proteins were extracted from the feeding stuffs with dilute alkali. This procedure, furthermore, introduces large amounts of sodium chloride into the hydrolysate, and Thimann [1926] has shown that precipitation of the bases under these conditions is not reliable.

The isolation method of Kossel & Kutscher [1900], as modified by Vickery & Leavenworth [1928], offers definite advantages but requires large amounts of material. Block [1934, 2] adapted the method to small quantities of protein and this has been used, together with the Van Slyke and the original Vickery & Leavenworth methods, by Miller [1935] for the analysis of grass proteins. An examination of his data shows that the values obtained by the Van Slyke method must be considered extravagant. There is thus reason to doubt the accuracy of most of the existing values for the lysine content of feeding stuffs and for this reason, and because of the importance of lysine in the diet of animals, a re-investigation of the subject has been made.

The isolation method of Block [1934, 2] was selected for preliminary studies. Experience showed, however, that even when all the precautions recommended by Miller [1935] are exercised, the method does not give reliable results with impure proteins. Its main faults are as follows.

(1) The precipitation of arginine and histidine as their silver salts, by introducing Ag^+ as silver nitrate, leaves NO_3^- in the lysine filtrate, and at a

later stage, when the acidified solution is concentrated to 2 ml., there is danger of lysine being attacked by the nitric acid present. (2) The N recovered from the phosphotungstate precipitate is assumed to belong exclusively to lysine and the theoretical quantity of picric acid required to form lysine picrate is added. This is unsatisfactory, since as much as 40 % may be due to other substances, and the excess picric acid may form the soluble lysine dipicrate. The procedure of Calvery [1929] is preferable. (3) The lysine picrate is not recrystallized. Even with pure protein the product does not explode above 250° and Vickery & Leavenworth [1928] stress the fact that recrystallization is necessary. The loss on recrystallization is about 4 %.

It was found that if ammonia was removed immediately after hydrolysis and the silver salts of arginine and histidine removed together at *p*H 14 somewhat more satisfactory results could be obtained, some of which are given in Table I.

Table I. *Analyses of certain feeding stuffs by a modification of Block's procedure*

Feeding stuff	Wt. g.	N %	Total N g.	Lysine picrate obtained g.	Lysine N as % of total N
Earthnut cake	6·000	7·60	0·4560	0·2000	3·3
Wheat gluten	3·025	12·59	0·3815	0·0750	1·5
Blood meal	6·098	13·68	0·8342	0·8084	7·4

All weights and N values are expressed in % of the air-dry feeding stuff.

The modified procedure finally adopted in the present work, however, embodies certain interesting new features. The protein or feeding stuff is hydrolysed in the normal way with HCl, the hydrolysate diluted to such a volume that the HCl concentration is about 5 % and phosphotungstic acid added until no further precipitate is obtained. Humin and ammonia are thus precipitated together with the bases. After standing, the precipitate is filtered off, washed and decomposed in the presence of mineral acid by amyl alcohol-ether according to the usual Van Slyke procedure. Ammonium phosphotungstate, however, is not affected by this treatment and remains suspended together with the humin in the aqueous phase, which, of course, contains the free bases. After complete removal of the phosphotungstic acid in the usual way by the mixed organic solvents the aqueous phase is centrifuged and we thus obtain a clear, nearly colourless, solution of the bases without having had to perform any of the following operations incidental to the normal Block procedure—(1) removal of the mineral acid used for hydrolysis, (2) concentration of the hydrolysate at any stage and (3) removal of ammonia as gas from an alkaline solution. The resulting gain in time and saving of labour needs no emphasis.

Arginine and histidine are next removed together as silver salts at *p*H 14 by adding silver oxide instead of silver nitrate, the filtrate freed from reagents and the lysine precipitated as picrate by the procedure of Calvery [1929]. On recrystallization the first (and main) crop of picrate explodes at 266–267° and the second at 250°, thus satisfying the more rigorous conditions of Vickery & Leavenworth [1928].

Experimental

4–5 g. of protein are hydrolysed with five times the weight of 20 % HCl for 36–40 hr., and the hydrolysate quantitatively transferred to a 250 ml. pyrex centrifuge bottle, using as little hot water as possible for washing. The bases are precipitated by the addition of a saturated solution of phospho-24-tungstic acid

(A.R.) in water until an excess is present. Water is then added, with stirring, until the final concentration of HCl is 5 % by weight and the mixture kept at room temperature overnight.

The precipitate is centrifuged (to obtain a good separation it is sometimes necessary to stir before centrifuging), and washed twice by whipping up with 25 ml. quantities of a 2·5 % phospho-24-tungstic acid solution in 5 % (by weight) H_2SO_4 and centrifuging. The phosphotungstates are decomposed by disintegrating with 50 ml. of water (to which has been added 1 ml. of conc. H_2SO_4) and a liberal quantity of amyl alcohol-ether [Van Slyke, 1915] and the mixture centrifuged. The clear liquids (both aqueous and organic) are poured into a separating funnel. The residue, which consists of the acid-insoluble humin, the humin precipitated by the phosphotungstic acid and ammonium phosphotungstate, is washed twice, as before, using smaller quantities of acidified water and amyl alcohol-ether, the washings being added to the mixed liquids in the separating funnel. After a few minutes the aqueous solution, which should be water clear, is drawn off and shaken out three times successively with amyl alcohol-ether. The combined amyl alcohol-ether solutions are shaken once with a small quantity of water acidified with H_2SO_4, and the latter solution washed once with a fresh quantity of the amyl alcohol-ether. The aqueous solutions are then combined and given a final washing with a small quantity of the amyl alcohol-ether.

With the blood meal samples it was found more satisfactory to decompose the phosphotungstates with 50 ml. of acidified water and 75 ml. of the amyl alcohol-ether. The organic liquid forms the bottom layer on centrifuging and the clear aqueous layer may be syphoned off. The amyl alcohol-ether and insoluble precipitate are washed by stirring up three successive times with 25 ml. quantities of acidified water and centrifuging; the aqueous washings being siphoned off as before.

The final aqueous solution is run into a 500 ml. flask, reduced *in vacuo* to a small volume and quantitatively transferred to a 250 ml. pyrex centrifuge bottle. It is then heated in a boiling water bath and solid silver oxide added, in small quantities at a time, with rapid stirring, until an excess of silver is present (brown ppt. with cold saturated baryta). An excess of silver is readily obtained by this method, which is much quicker than shaking the cold aqueous solution with a suspension of silver oxide in water [Vickery & Leavenworth, 1928], and the final volume, for 5 g. of protein, is less than 200 ml. The solution is cooled and made distinctly alkaline to Nile blue, or alizarin yellow S, by the addition of warm saturated aqueous baryta. The precipitate is centrifuged and washed twice with small amounts of cold saturated aqueous baryta. The centrifugate and washings are transferred to a suitable Büchner flask, made acid to Congo red and the silver precipitated with H_2S under pressure. The mixture of $BaSO_4$ and Ag_2S is centrifuged and thoroughly washed with water. The clear aqueous solution is then concentrated *in vacuo* and made up to 100 ml., 1 ml. aliquots being withdrawn for estimation of total N.

The remaining solution is transferred to a 250 ml. centrifuge bottle, the major part of the H_2SO_4 removed as $BaSO_4$, the precipitate washed and the filtrate and washings concentrated to a small volume *in vacuo* in the presence of a small excess of $BaCO_3$. The $BaCO_3$ and $BaSO_4$ are then removed by filtration through a No. 42 Whatman filter and the filtrate concentrated *in vacuo* to 2 ml. The detachable head of the flask and the capillary are then washed down with 5–10 ml. of 93 % alcohol, one or two drops of water being added if the mixture becomes more than faintly turbid. For each mg. of N present in solution, half the quantity of picric acid required to form the lysine picrate is then added. Crystals

should form on stirring the solution, which is left in the ice chest overnight. The lysine picrate is filtered off under reduced pressure on a sintered glass crucible, washed with cold absolute alcohol and the filtrate tested for unprecipitated lysine by addition of a further quantity of picric acid dissolved in warm absolute alcohol [Calvery, 1929]. The crude lysine picrate is filtered off on the same sintered glass crucible as the first crop, and the combined crops dried and weighed. The material is then dissolved in hot water, the solution sucked through the crucible and concentrated until crystals appear on the surface. The solution is cooled in the ice chest, the lysine picrate filtered off, washed with absolute alcohol, dried in a steam oven and weighed. The filtrate and washings are concentrated to a small volume, cooled and the crystals collected as before. The final volume of the mother liquor from this crop is measured, and a correction of 3·4 mg. per ml. applied for the solubility of the lysine picrate. The first crop should explode at a temperature of 266–267° and the second crop above 250° [Vickery & Leavenworth, 1928].

The data given in Table II were obtained by this method. The casein used was a sample of Kahlbaum's Hammarsten casein, and contained 14·5 % N (dry weight). Blood meal A was a sample used in a study of the relationship between

Table II. *Lysine content of casein and blood meals*

	Wt. of sample (air-dried) g.	Total N g.	Wt. of lysine picrate		Solubility correction g.	Lysine N as % of total N
			1st crop g.	2nd crop g.		
Casein	5·8620	0·8180	0·792	0·0690	0·0170	8·03
,,	5·2790	0·7364	0·702	0·0410	0·0170	7·74
Blood meal, sample A	3·7520	0·4724	0·437	0·0560	0·0180	8·00
,, ,, B	4·0660	0·5120	0·453	0·0773	0·0153	7·97
,, ,, C	5·0770	0·7362	0·786	0·1040	0·0136	9·18
,, ,, D	4·9865	0·7050	0·778	0·0400	0·0136	8·83

milk yield and the lysine content of feeds [Bartlett, 1936]. This meal contained 12·6 % N and was probably dried at a high temperature during the course of manufacture, for it contained a very large quantity of extremely hard particles which were most difficult to hydrolyse. Samples C and D were prepared from blood clot. Sample C contained 14·5 % N, was water-soluble and was spray-dried at 134° F. Sample D contained 14·1 % N and consisted of the "fines" screened from meal dried in a steam-jacketed pan at 220° F. The values obtained for the lysine content of casein agree very well with that (7·72 %) obtained by Vickery & White [1933].

The new method is probably the shortest that can be devised for the determination of lysine, using phosphotungstic acid as a precipitant. The time required for a complete analysis, in duplicate, is about 3 days. The method when applied to a feed containing a low percentage of lysine (wheat gluten) was not successful. It is believed that this is due to the very high percentage of proline in the feed. The proline would be precipitated with the bases, and, since it would not be removed as the silver salt, would be present in the final lysine fraction and thus prevent the formation and isolation of pure lysine picrate.

The values found for the lysine content of the blood meals vary somewhat, but in no case approach the high value obtained by Morris [1934] for a sample of blood meal analysed by the Van Slyke procedure. There is little doubt that the treatment of blood meal in course of manufacture will determine its value as a source of protein and lysine. As pointed out above, some of the meals were

extremely hard and almost impossible to reduce to a fine powder in a heavy mortar, and required prolonged digestion with 20% HCl to effect complete hydrolysis. It is likely that such meals would be of little value as feeding stuffs, even though they were shown to be rich in lysine by chemical analyses.

SUMMARY

1. A shorter method for the determination of lysine in proteins is described, and is applied to the analyses of blood meals.

2. Values found for lysine in blood meals by the isolation method are considerably below published values obtained by the Van Slyke procedure.

I wish to thank Prof. A. C. Chibnall and Prof. H. D. Kay for their valuable advice and encouragement during the course of this work.

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CLII. ASCORBIC ACID AND PHOSPHATASE ACTIVITY

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In a recent communication Thannhauser *et al.* [1937] claim to have shown that ascorbic acid is an intense activator of serum phosphatase. We have been unable to confirm their claim.

In preliminary experiments, Thannhauser's published technique was adopted. The results of some typical experiments are shown in Table I. It will be seen that the presence of ascorbic acid produced no appreciable change in the amount of phosphatase hydrolysis.

Table I. *Phosphatase hydrolysis of phosphoric esters in the presence of ascorbic acid*

Enzyme	Units of phosphatase activity: Without ascorbic acid	Units of phosphatase activity: With ascorbic acid	Method
β-Glycerophosphate, veronal buffer *p*H 8·9, Bodansky [1933] units			
Serum	6·4	8·4	Thannhauser *et al.*: 1 hr. at 37°, Briggs [1922]
Serum	7·0	7·0	P-method—reducing agent quinol
Serum	10·0	8·0	
Serum	14·0	17·0	
Serum	8·0	12·0	
Plasma	2·0	2·0	
Serum	2·0	2·0	(α-Glycerophosphate)
Serum	6·0	4·0	
Plasma	2·0	2·0	Ammon & Hinsberg [1936] P-method—reducing agent ascorbic acid
Plasma	4·9	4·7	
Plasma	5·0	5·0	P-precipitation method [Delory, 1938]
Serum	6·0	5·0	
Phenylphosphate, sodium carbonate—bicarbonate buffer *p*H 9·9, King-Armstrong units			
Serum	8·0	8·0	45 min. 37°, King's [1932] P-method—reducing agent aminonaphtholsulphonic acid
Serum + bile	44·0	47·0	
Serum + bile	40·0	38·0	
Serum + faeces	500·0	515·0	
Plasma	6·2	6·7	P-precipitation method [Delory, 1938]
Plasma	20·0	20·0	

The effect of using sodium α-glycerophosphate, sodium phenylphosphate, and sodium *p*-nitrophenylphosphate as substrate was also studied. In the case of sodium α-glycerophosphate, Thannhauser's technique was used, the α-glycerophosphate being substituted for the β-glycerophosphate.

Experimental

In view of Thannhauser's insistence on the necessity for speed to minimize the loss of ascorbic acid through oxidation, great care was taken to keep the time which elapsed between the addition of the several reagents (buffer, substrate etc.) down to a minimum. The ascorbic acid solution was prepared immediately before use.

Ascorbic acid reduces the phosphomolybdic acid formed during the course of the colorimetric phosphate determination to give a blue colour. A given amount of phosphate, in the Briggs [1922] procedure with quinol as the reducing agent, yields more blue colour in the presence of ascorbic acid than is obtained in its absence, the amount of extra colour depending on the amount of ascorbic acid present. As Thannhauser has shown, it is necessary to make a correction for this increase in colour. This procedure is not entirely satisfactory because the amount of ascorbic acid remaining in the tests (after 1 hr. at 37° and *p*H 8·9) is uncertain. Methods were therefore devised to overcome this difficulty. The phosphate was precipitated away from the remaining ascorbic acid solution in one set of experiments, while in another set, an excess of ascorbic acid was used as reducing agent, according to the colorimetric phosphate method of Ammon & Hinsberg [1936], no other reducing agent being added.

In the precipitation experiments, the hydrolysis was carried out in the usual way, and an amount of each of the trichloroacetic acid filtrates taken for the phosphate estimation according to the method described in the appended note [Delory, 1938]. Results of typical experiments are shown in Table I.

Attempts to use the King & Armstrong [1934] phenylphosphate method for phosphatase determination proved unsuccessful since the ascorbic acid interfered with the phenol reagent. It was therefore necessary to modify the method slightly and to determine the free phosphate produced instead of the phenol. Four tubes were set up as in the following scheme:

	ml. diluted serum or plasma (1 : 10)	ml. water	ml. ascorbic acid solution (5 mg. per ml.)	ml. *N*/20 NaOH
Tubes 1 and 2	1	1·45	1	0·55
Tubes 3 and 4	1	3·00	—	—

The tubes were placed in the water bath at 37°, and when the contents had attained the temperature of the bath (2 min.), 6 ml. of *M*/200 phenylphosphate in *M*/20 sodium carbonate-bicarbonate buffer (previously warmed at 37°) were added to each tube. The final *p*H in all the tubes was 9·9 (optimum for phenylphosphate hydrolysis). Immediately after addition of the substrate 2·5 ml. of 20% trichloroacetic acid were added to tubes 1 and 3 which served as controls. Tubes 2 and 4 were kept at 37° for ¾ hr. and were then treated with 2·5 ml. of trichloroacetic acid. The acid mixtures were kept at 0° for 30 min. to allow the precipitates to coagulate before filtering. 10 ml. of the trichloroacetic acid filtrates were used for colorimetric phosphate determination [King, 1932]. In this method, as in that of Briggs, the amount of blue colour was affected by the presence of ascorbic acid. The figures had, therefore, to be corrected in terms of the increased colour produced on adding known amounts of ascorbic acid to standard solutions. Experiments were also conducted with the precipitation procedure for liberated phosphate.

The results are expressed as units equal to mg. P liberated by 100 ml. of serum or plasma. The ¾ hr. incubation period was chosen because three times as much phenol as phosphorus by weight is liberated in the hydrolysis, and the King-Armstrong unit is defined in terms of the mg. phenol liberated in ¼ hr. at *p*H 9·9 [King *et al.* 1937].

Experiments were carried out to study the rate of oxidation of ascorbic acid under the conditions of the phosphatase hydrolysis. Two tubes were set up consisting of buffer, substrate, serum and ascorbic acid plus an equivalent amount of *N*/20 NaOH. To one of these tubes trichloroacetic acid was added

immediately, while to the other, the trichloroacetic acid was added after 45 min. at 37°. The filtrates were titrated with 2:6-dichlorophenolindophenol. In two typical experiments 5·1 and 5·4 mg. of ascorbic acid were recovered from the two tubes to which trichloroacetic acid was immediately added, and 4·1 and 3·8 mg. from those which were incubated for 45 min.

Experiments with dehydroascorbic acid produced by bubbling oxygen through a solution of ascorbic acid at *p*H 7, in the presence of a trace of ferrous sulphate [Thannhauser *et al.*] failed to affect the phosphatase activity.

The accurate determination of inorganic phosphate (in these experiments) was only possible by the use of a photometer or a photoelectric colorimeter. The colours produced in the Briggs method by the amounts of phosphate present in tests and controls were altogether too pale to allow of colorimetric comparison with an ordinary Duboscq type of instrument. The control in the Thannhauser procedure has only the amount of phosphorus represented in half the filtrate from 0·1 ml. of blood serum, i.e. about 1–2 μg. inorganic P, and in a test representing 10 units of phosphatase activity the inorganic phosphate is increased by only 5 μg. These very small amounts yield such pale colours in the final volume of 25 ml. used by Thannhauser in the Briggs method, that they can only be estimated by photometric measurement of the light absorbed by a considerable thickness (e.g. 3 cm.) of coloured solution. The measurements were made in a Zeiss Pulfrich photometer, a Millikan photoelectric colorimeter, and a Leitz "absolute colorimeter". But it was also desired to make the determinations of hydrolysed phosphate by ordinary colorimetric means. For this purpose the experiments were repeated using such larger amounts of serum or plasma as were capable of giving sufficient inorganic phosphate to be determined accurately in a Duboscq colorimeter. Appropriate buffer mixtures were used to give a final *p*H of 8·9 for the glycerophosphate substrates, and 9·9 for the phenylphosphate. The enzyme action was stopped and the proteins precipitated by amounts of trichloroacetic acid sufficient to give a final concentration of 4%. The results of several experiments are given in Table II.

Table II. *Effect of ascorbic acid on phosphatase activity*

(5 mg. ascorbic acid for each 0·1 ml. of serum or plasma used.)

Enzyme	Units of phosphatase activity: Without ascorbic acid	Units of phosphatase activity: With ascorbic acid	Phosphate method
	Bodansky units		
Serum (0·2 ml.)	10·0	9·0	Briggs [1922]
Serum (0·2 ml.)	5·0	5·0	,,
Serum (0·2 ml.)	8·0	12·0	,,
Plasma (0·5 ml.)	3·6	3·6	,,
Plasma (0·4 ml.)	5·0	5·0	Ammon & Hinsberg
Plasma (0·4 ml.)	1·8	1·5	[1936]
Plasma (0·2 ml.)	12·0	13·0	Delory [1938]
	King-Armstrong units		
Serum (0·2 ml.)	4·0	3·0	King [1932]
Plasma (0·2 ml.)	4·3	4·2	,,
Plasma (0·2 ml.)	5·0	5·0	Ammon & Hinsberg,
Plasma (0·4 ml.)	4·0	4·0	[1936]
Plasma (0·2 ml.)	10·0	10·0	Delory [1938]

The activity of plasma phosphatase with *p*-nitrophenylphosphate was studied in the presence and absence of ascorbic acid. Free *p*-nitrophenol is

much more highly coloured in alkaline solution than is its phosphoric ester. It was possible to follow the hydrolysis by studying the increase in yellow colour due to the liberation of the free phenol.

Equal amounts of substrate and buffer solution (*p*H 10) were placed in two similar tubes. 5 mg. of ascorbic acid were titrated with *N*/10 NaOH to phenolphthalein. The volume of *N*/10 NaOH required for neutralization was added to one of the tubes followed by 5 mg. of ascorbic acid. To the other tube was added enough water to produce the same total volume. To both tubes were added 0·1 ml. of phosphatase solution. The development of colour in the two tubes was then followed by taking measurements with the photoelectric colorimeter.

Reference to Table III shows that ascorbic acid had no effect on the phosphatase activity with this substrate.

Table III. *Hydrolysis of* p-*nitrophenylphosphate*

(5 ml. *M*/1000 substrate in carbonate-bicarbonate buffer.)

		Reading of photoelectric colorimeter (min.)			
		0	15	30	45
Plasma	Control	5	11	17	—
	+7 mg. ascorbic	5	11	17	—
Serum	Control	6	10	12	18
	+7 mg. ascorbic	6	9	11	18
Purified kidney phosphatase	Control	4	21	30	36
	+4 mg. ascorbic	5	21	29	35
,,	Control	8	18	23	30
	+3 mg. ascorbic	8	17	20	28

In phosphatase determinations on the sera from cases of generalized bone disease and obstructive jaundice Thannhauser *et al.* could find little or no increase in activity in the presence of ascorbic acid. They concluded that the phosphatase in the blood of those cases was already optimally activated, and that the high phosphatase values characteristically encountered in these diseases are due to an increased enzyme activity rather than to an increased amount of enzyme. In the absence of any confirmation of the activation by ascorbic acid of the phosphatase in sera of normal persons, no support can be given to this contention.

Summary

The influence of ascorbic acid on the enzymic hydrolysis of the phosphoric esters commonly used for phosphatase determinations has been studied. No appreciable effect on the activity of serum phosphatase with these substrates can be demonstrated. It is concluded that ascorbic acid does not activate phosphatase. No support is, therefore, found for the contention of Thannhauser *et al.* [1937] that the high serum phosphatase values encountered in certain diseases are due to the presence of an activator in the serum rather than to an accumulation of the enzyme itself.

The authors gratefully acknowledge the gift of samples of ascorbic acid and sodium β-glycerophosphate from Prof. Thannhauser, who thus made it possible for them to repeat the experiments with the original materials.

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A NOTE ON THE DETERMINATION OF PHOSPHATE IN THE PRESENCE OF INTERFERING SUBSTANCES

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THE colorimetric determination of phosphate is often difficult or impossible in the presence of interfering substances. For this reason it has been found convenient to precipitate the phosphate away from the interfering substance and to determine it colorimetrically in the redissolved precipitate. Molybdic acid and an organic base are frequently used as precipitants. But in the presence of reducing substances such as ascorbic acid, this precipitation may be impossible, since these substances reduce the phosphomolybdate.

It has been found possible to precipitate completely the inorganic phosphate as $Ca_3(PO_4)_2$ in amounts as small as 0·01 mg. P by entrainment with light magnesium carbonate powder ($MgCO_3$). The centrifuged precipitate is washed, dissolved in perchloric acid, and the colorimetric determination carried out in the usual way.

EXPERIMENTAL

Measured amounts of phosphate solution are introduced into centrifuge tubes graduated at 15 ml. The contents of the tubes are neutralized (where necessary) to phenolphthalein by the dropwise addition of concentrated ammonia solution. An extra 0·2 ml. of the same solution is then added. After the addition of 1 ml. of 2·5 % calcium chloride solution and 1 ml. of a 0·5 % suspension of "light" magnesium carbonate in water, the tubes are shaken and allowed to stand for 30 min. They are shaken again and then centrifuged. The supernatant fluids are carefully poured off and discarded. The tubes are allowed to drain and the precipitates washed with 5 ml. of 2 % ammonia solution (saturated with calcium phosphate). The tubes are centrifuged and drained as before. The precipitates are dissolved in 1·1 ml. of 60 % perchloric acid. About 10 ml. of water are added, followed by 1 ml. of 5 % ammonium molybdate solution and 0·5 ml. of 1:2:4-aminonaphtholsulphonic acid solution. Water is added to bring the total volume to 15 ml. After standing for 10 min., the blue solutions are read in the colorimeter against standard solutions prepared from suitable amounts of a dilute standard phosphate solution together with 1 ml. of 60 % perchloric acid, etc., in 15 ml. volumetric flasks [King, 1932].

The table shows recovery of phosphorus from (1) simple solution; (2) 2·5% trichloroacetic acid; (3) solutions containing 5 mg. ascorbic acid; (4) 2·5% trichloroacetic acid solutions containing 5 mg. ascorbic acid; and (5) solutions containing 5 mg. ascorbic acid, trichloroacetic acid and 1 ml. of 5% sodium β-glycerophosphate.

If the amount of phosphate to be recovered is very small, it may be desirable to concentrate the colour produced by making the solution of the precipitate up to a smaller final volume. For this purpose the precipitate is dissolved in 0·25 ml. perchloric acid followed by 2·5 ml. water, 0·2 ml. ammonium molybdate and 0·1 ml. aminonaphtholsulphonic acid, making a total volume of 3 ml.

Table I. *Recovery of inorganic phosphate by precipitation with calcium*

(Figures represent mg. P.)

(1) Phosphate precipitated from simple solution				
P present	0·025	0·050	0·075	0·100
P recovered	0·025	0·048	0·080	0·100
(2) Phosphate precipitated from trichloroacetic acid solution				
P present	0·010	0·020	0·030	0·040
P recovered	0·010	0·018	0·026	0·037
(3) Phosphate precipitated from ascorbic acid solution				
P present	0·010	0·050	0·070	0·100
P recovered	0·010	0·048	0·067	0·096
(4) Phosphate precipitated from trichloroacetic and ascorbic acid solution				
P present	0·020	0·050	0·070	0·100
P recovered	0·018	0·050	0·071	0·100
(5) Phosphate precipitated from solution containing β-glycerophosphate trichloroacetic acid and ascorbic acid				
P present	0·020	0·050	0·070	0·100
P recovered	0·020	0·049	0·070	0·099

REFERENCE

King (1932). *Biochem. J.* **26**, 292.

CLIII. THE EFFECT OF ASCORBIC ACID ON β-GLYCEROPHOSPHATE

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In a previous paper [1937] we reported that ascorbic acid activated serum phosphatase, as shown by the increase in inorganic phosphate in the presence of sodium β-glycerophosphate. The accompanying paper by King & Delory [1938] reports experiments similar to ours but in which no activating effect by ascorbic acid was observed. Dr King very kindly informed us of his inability to repeat our experiments and has graciously permitted us to report our findings as to the cause of the discrepancy. We are now able to explain why we obtained the results which we reported previously. In the experiments of our previous paper, we allowed serum phosphatase, β-glycerophosphate, ascorbic acid and buffer (*p*H 8·9), to incubate for 1 hr. at 37°. At the end of this period, trichloroacetic acid was added to stop enzymic activity. After 30 min. the precipitate was filtered. The solutions were allowed to stand overnight before the determination of phosphate was carried out. King & Delory's experiments differed from ours in that they determined the phosphate immediately after addition of the trichloroacetic acid and the removal of the precipitate. We believed that our procedure was justifiable because we had obtained no appreciable acid hydrolysis of β-glycerophosphate over a 48-hr. period with trichloroacetic or mineral acids at *p*H 1, in the absence of ascorbic acid (Table I). It is to be regretted that we did not run a control experiment comparing the splitting of phosphate in the presence of ascorbic acid after the 24-hr. period, for to our great surprise we have found that β-glycerophosphate is hydrolysed directly by ascorbic acid at

Table I. *The effect of different acids on β-glycerophosphate*

Temperature, 37°; 50 mg. β-glycerophosphate; 5 mg. ascorbic acid; *p*H determined electrometrically; total volume, 20 ml.

	Time (hr.)	*p*H	No ascorbic acid mg. P	Ascorbic acid, 5 mg. mg. P
β-Glycerophosphate + trichloroacetic acid (0·15 *N*)	24	1·1	0·0027	0·095
	48	1·1	0·0030	0·118
β-Glycerophosphate + acetic acid (10 *N*)	24	1·1	0·0035	0·105
	48	1·1	0·0035	0·135
β-Glycerophosphate + hydrochoric acid (0·1 *N*)	24	1·1	0·0035	0·004
	48	1·1	0·0038	0·005
β-Glycerophosphate + sulphuric acid (0·1 *N*)	24	1·1	0·0029	0·044
	48	1·1	0·0040	0·086

acid *p*H, the amount of splitting increasing with time. As Table I shows, ascorbic acid at *p*H 1 increases markedly the amount of phosphate split from β-glycerophosphate. This effect seems to be equally great with both trichloroacetic and acetic acids. It is considerably less with sulphuric acid, and seems to occur

hardly at all with hydrochloric acid. This direct action of ascorbic acid on β-glycerophosphate was not known and therefore in our published experiments an increased splitting of this substrate, in the presence of serum phosphatase and vitamin, was interpreted as an activation of the enzyme by the vitamin. The theory of ascorbic acid activation of serum phosphatase, advanced in our previous paper, must be abandoned, because it is now evident that the increased phosphorus values reported were brought about by the direct action of ascorbic acid on β-glycerophosphate in acid medium.

The suggestion that increased colour in our colorimetric determination might be due to some other factor than an increased amount of free phosphate led us to perform gravimetric determinations for free phosphate, using ten times as much material as that used in colorimetric work. The results were in fair agreement with those obtained colorimetrically.

In our previous paper it is mentioned that ascorbic acid fails to activate lecithin, sphingomyelin or adenosinephosphoric acid, which in the light of our present knowledge indicates that vitamin C is unable to act directly on these substrates. This observation was extended to include monophenylphosphate. We do not desire to put forth at this time a definite explanation as to why ascorbic acid splits β-glycerophosphate and fails to act on the other substrates investigated. To us it seems possible, however, that ascorbic acid, characterized by its high redox potential, affects the glycerol portion of the phosphoric ester and in this way may bring about splitting of the ester compound.

With regard to the theory advanced by us that increased values in such conditions as Paget's disease and obstructive jaundice are due to an activated phosphatase rather than to an increased amount of the enzyme, it may be mentioned that we have confirmed this theory by other observations made on serum with normal, and serum of high phosphatase activity, results of which will be published shortly.

In Table I it can be seen that no appreciable splitting of the phosphoric ester takes place in the presence of the acids themselves. Even after a hydrolysis period of 3 weeks this was still found to be the case. When ascorbic acid was added to these solutions containing acid and phosphoric ester its ability to split the ester was greatly influenced by the particular acid present. Hydrochloric acid completely overcomes the action of ascorbic acid, while sulphuric acid interferes to a great degree, and even trichloroacetic acid to a lesser degree, acetic acid having the least influence on the action of the vitamin.

Table II contains the results obtained when the concentrations of materials were increased ten times. The diminishing effect of trichloroacetic acid is evident.

Table II. *The effect of ascorbic acid on β-glycerophosphate using increased concentrations (ten times usual concentration)*

Temperature, 37°; 500 mg. β-glycerophosphate; 50 mg. ascorbic acid; 10 ml. 10% trichloroacetic acid; total volume 20 ml.

(*a*) β-Glycerophosphate + trichloroacetic acid *p*H = 1·1.
(*b*) β-Glycerophosphate + trichloroacetic acid + ascorbic acid *p*H = 1·1.
(*c*) β-Glycerophosphate + ascorbic acid *p*H = 3·1–6·9.

Time in thermostat days	*a* mg. P	*b* mg. P	*c* mg. P
2	0·003	0·250	0·570
10	0·006	0·500	1·550
20	0·011	1·250	1·675

SUMMARY

1. The direct action of ascorbic acid alone on sodium β-glycerophosphate is reported.

2. The failure of hydrochloric, sulphuric, trichloroacetic, and acetic acids to hydrolyse β-glycerophosphate at pH = 1 has been shown.

3. The influence of hydrochloric, sulphuric, trichloroacetic and acetic acids, on the action of ascorbic acid, with regard to its splitting of β-glycerophosphate, is demonstrated.

4. The theory of ascorbic acid activation of serum phosphatase, outlined in a previous paper, is abandoned in the light of the present knowledge of ascorbic acid action on sodium β-glycerophosphate.

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CLIV. THE ULTRAVIOLET ABSORPTION AND POTENTIOMETRIC TITRATION CURVES OF HUMAN SERUM PROTEINS AND SOME OTHERS

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(*Received 25 April 1938*)

This work is a continuation of that of Holiday [1936] and of Ogston [1936]. The work of Tiselius [1937], on the separation of serum proteins by electrophoresis which has appeared since this work was begun, has explained some of the earlier results and in some degree reduced the value of ours. It is now clear from his investigations and from those of McFarlane [1935] that homogeneous preparations of serum proteins are not obtained by precipitation with ammonium sulphate.

In our earlier work some degree of variation was found in the quantitative properties of different specimens of normal horse serum proteins. Our first object was to establish whether these variations are due to causes other than detailed differences in the method of separation; measurements were therefore made on the pseudoglobulins of the serum of a number of normal persons, separated by a standardized technique. As the opportunity arose, measurements were made also on some preparations of normal serum albumin, on serum and urinary proteins of two nephrotic patients, on some purified antibodies to types II and III pneumococcus and on a solution of seroglycoid prepared by Dr L. F. Hewitt.

Ultraviolet absorption spectra were measured as described by Holiday [1936]. Potentiometric titration curves were obtained by the method of Ogston [1936]: at least two concordant runs were made over each limb of the curve. Discordant results were very rare. The number of primary amino groups was obtained by potentiometric titration in presence of 4 % formaldehyde.

Materials

(1) *Normal pseudoglobulin* in serum obtained from 30 ml. of human blood was thrice precipitated from 20-fold dilution by 40 % saturated ammonium sulphate, allowing minimum time for floculation: the time of contact with strong ammonium sulphate never exceeded 8 hr. The separated globulin was dialysed in rotating cellophane sacs against running distilled water until negative to the Nessler test for ammonia, performed on the protein solution. The precipitated euglobulin was removed by filtration.

(2) *Normal albumin.* A specimen of normal human albumin was prepared from human blood by two successive precipitations by 80 % saturated ammonium sulphate after removal of the globulin, and dialysis against distilled water. Of three specimens of crystalline horse serum albumin, one was prepared by Dr Wolff in these laboratories; one was kindly sent to us by Dr L. F. Hewitt; the third was prepared by the method of Hewitt [1936] and had a carbohydrate content of less than 0·1 %.

[1] Owen Williams Scholar. [2] Freedom Research Fellow.

(3) *Nephrotic proteins* were prepared from the serum and urine of two patients. From serum, the pseudoglobulin was obtained by the method given above: two fractions of albumin were obtained by 66% and 80% saturated ammonium sulphate respectively. From urine, after thorough dialysis against tap water, two pseudoglobulin fractions were obtained by 40 and 50% saturated ammonium sulphate, and albumin by 60% saturated ammonium sulphate.

(4) Concentrates of protein antibodies to types II and III pneumococcus were prepared by Prof. J. R. Marrack from serum of immunized horses; the antibodies were obtained from their sera by elution of the specific precipitates.

Results

(1) *Tyrosine and tryptophan.* Values of the tyrosin and tryptophan contents of the proteins derived from their ultraviolet absorption curves [Holiday, 1936] are given in Table II.

Corrected values for the extinction coefficients of tyrosine and tryptophan were used in the calculation, based on a rematching of spectrograph plates with

Table I. *Molecular extinction coefficients of tyrosine and tryptophan*

In $N/10$ NaOH $\epsilon \times 10^{-3}$

Wave-length mμ ...	260	265	270	275	280	285	290	295	300	305	310	315
Tyrosine	1·75	1·14	1·08	1·24	1·54	1·96	2·24	2·26	1·89	1·14	0·471	0·182
Tryptophan	3·10	3·80	4·45	5·15	5·43	4·65	4·35	2·15	1·10	0·45	0·140	0·051

In $N/10$ HCl $\epsilon \times 10^{-3}$

Wave-length mμ ...	260	265	270	275	280	285	290	295	300	305	310	315
Tyrosine	0·55	0·86	1·15	1·24	1·08	0·55	0·07	—	—	—	—	—
Tryptophan	3·90	4·65	5·23	5·37	5·46	4·45	3·72	1·35	0·57	0·10	—	—

a photoelectric microphotometer: these corrected values are given in Table I. The revised equations for the analysis of mixtures are:

$$M_{\text{tyrosine}} = (0{\cdot}99\ E_{305} - 0{\cdot}032\ E_{280}) \times 10^{-3}.$$
$$M_{\text{tryptophan}} = (0{\cdot}207\ E_{280} - 0{\cdot}280\ E_{305}) \times 10^{-3}.$$

The following is a more reliable method of correcting for haze or pigment than that given formerly:

(1) $M_{\text{tryptophan}}$ is obtained from the nomogram.

(2) From this E_{305} (tryptophan) is calculated $= M_{\text{tryptophan}} \times 0{\cdot}45 \times 10^3$.

Table II. *Amino, tyrosine, tryptophan: milliequivalents per g. protein*

Protein	Amino	Tyrosine	Tryptophan
Mean of 13 normals	0·449	0·380	0·150
Standard deviation	0·030	0·040	0·013
A 13	0·600	—	—
Horse crystalbumin (Hewitt)	0·680	0·278	0·016
Horse crystalbumin	0·670	0·290	0·005
Crystalbumin, horse albumin	0·650	—	—
Horse seroglycoid (Hewitt)	0·415	0·320	0·114
H 40 (serum)	0·415	0·365	0·164
H 50 (urine)	0·530	—	—
I 40 (urine)	0·500	0·304	0·168
I 50 (urine)	0·395	0·315	0·163
H 66 (serum)	0·640	0·304	0·080
H 80 (serum)	0·680	0·392	0·033
H 60 (urine)	0·570	0·355	0·020
Anti type II	0·500	0·410	0·103
Anti type II	0·590	0·470	0·160
Anti type III	0·410	0·380	0·160

Table III. *Titre from* p*H 7: milliequivalents per g. protein*

*p*H ...	3	3·5	4	4·5	5	5·5	6	6·5	7	7·5	8	8·5	9	9·5	10	10·5	11
Normal pseudoglobulins																	
Mean of 13	0·684	0·507	0·347	0·231	0·151	0·102	0·062	0·028	0	0·026	0·048	0·069	0·094	0·135	0·206	0·307	0·436
Standard deviation	0·029	0·020	0·020	0·014	0·013	0·008	0·005	0·003	0	0·003	0·003	0·006	0·006	0·009	0·011	0·014	0·025
Normal albumins																	
A 13 (human)	1·214	0·959	0·667	0·431	0·272	0·175	0·105	0·049	0	0·050	0·090	0·130	0·172	0·231	0·327	0·472	0·679
Horse seroglycoid (Hewitt)	0·863	0·678	0·503	0·373	0·268	0·186	0·118	0·056	0	0·040	0·077	0·111	0·137	0·182	0·261	0·387	0·574
Mean of three horse crystalbumins	1·268	1·003	0·668	0·406	0·244	0·151	0·092	0·044	0	0·051	0·105	0·163	0·208	0·260	0·340	0·463	0·689
Nephrotic proteins																	
Pseudoglobulins:																	
H 40 (serum)	0·742	0·565	0·400	0·264	0·167	0·112	0·064	0·026	0	0·028	0·055	0·076	0·098	0·140	0·207	0·323	0·452
H 50 (urine)	0·715	0·549	0·380	0·247	0·165	0·102	0·057	0·027	0	0·023	0·051	0·074	0·108	0·158	0·245	0·385	0·563
I 40 (urine)	0·753	0·580	0·405	0·278	0·182	0·100	0·056	0·028	0	0·027	0·056	0·092	0·130	0·187	0·261	0·358	0·495
I 50 (urine)	0·732	0·560	0·386	0·251	0·162	0·102	0·061	0·029	0	0·028	0·052	0·077	0·108	0·151	0·213	0·313	0·446
Albumins:																	
H 66 (serum)	1·036	0·805	0·530	0·325	0·215	0·146	0·090	0·043	0	0·046	0·087	0·127	0·171	0·231	0·318	0·459	0·680
H 80 (serum)	1·160	0·880	0·583	0·368	0·241	0·157	0·105	0·051	0	0·043	0·086	0·129	0·175	0·240	0·331	0·468	0·635
H 60 (urine)	1·238	0·996	0·708	0·468	0·296	0·182	0·105	0·050	0	0·058	0·112	0·153	0·200	0·275	0·387	0·537	0·762
Antibody proteins																	
Anti type II	1·050	0·860	0·643	0·435	0·260	0·140	0·058	0·018	0	0·022	0·043	0·069	0·098	0·150	0·248	0·395	0·615
Anti type II	0·855	0·705	0·525	0·340	0·195	0·113	0·062	0·024	0	0·021	0·040	0·072	0·114	0·190	0·320	0·515	0·825
Anti type III	0·778	0·638	0·468	0·323	0·198	0·121	0·066	0·027	0	0·022	0·040	0·057	0·080	0·122	0·207	0·325	0·472

(3) Since tyrosine in neutral solution has no absorption at 305 mμ the difference between E_{305} (tryptophan) and the extinction at 305 mμ of a neutral or acid solution of protein represents absorption due to haze or pigment (E_{pig}).

(4) Assuming that E_{pig} is the same at 305mμ and at 280 mμ and the same in acid and alkaline solutions, correction is made by subtracting E_{pig} from the extinctions of the protein in alkaline solution at these wave-lengths: from the corrected extinctions M_{tyrosine} and $M_{\text{tryptophan}}$ are calculated.

(2) *Titration curves.* The titration curves are expressed in Table III as titres from *p*H 7 in milliequivalents per g. protein. In Table II are given the free amino groups in milliequivalents per g. protein.

Discussion

Tiselius [1937] has shown that globulins separated by ammonium sulphate precipitation are mixtures, with the α-globulin predominating in "pseudoglobulin": he states (p. 1476, line 27) that his fractions differ in light absorption. Since their mobilities differ, their titration curves may differ. McFarlane [1935] found that pseudoglobulin preparations obtained by precipitation are somewhat heterogeneous in the ultracentrifuge. It is therefore not surprising that variations are found in the quantities which we have measured. Nevertheless we believe that our mean results represent fairly well the properties of normal pseudoglobulins prepared by our methods, and that, in lack of general availability of the electrophoretic method of separation, comparisons with these of values obtained with other proteins may be of some value.

There is no correlation with age or blood group of the variations found; comparison of these with our previous results for horse serum pseudoglobulin does not suggest any significant differences between them. The pseudoglobulins from the serum and urine of nephrosis give absorptions and titration values which vary somewhat from the normals, but it is doubtful whether these differences are sufficient to show with certainty that they are constitutionally different.

The values obtained for the antibodies should strictly be compared with those of horse serum euglobulin whose solubility properties they imitate, but reliable results for comparison are not available [Ogston, 1936]. However, it seems clear that the antibodies to types II and III pneumococcus are different from each other and that both are considerably different from serum globulin. Type II antibody shows a greater amino-titre than normal. Type III antibody resembles pseudoglobulin except in showing higher titres below *p*H 5, indicating a greater number of carboxyl groups. Neither antibody solution was completely precipitable by specific polysaccharide.

The two preparations of crystalbumin show closely concordant properties and there is no reason to doubt that our results represent the behaviour of this protein. The cruder preparation of human serum albumin differs from these to some extent, but since it was not crystallized, it probably contains seroglycoid: its titration curve accords with this.

Comparison with normals of the serum and urinary albumin of nephrosis, shows that the latter differ from normal and from each other: we regard these differences as significant.

The results obtained with seroglycoid are interesting since both the content of tyrosine, tryptophan and amino groups and the form of its titration curve suggest the properties of a globulin rather than of albumin. Comparison of the titration curves of seroglycoid and of crystalbumin suggest that, if their isoelectric points do not differ much, electrophoretic separation will be easy only at a *p*H below 4·5 or above 9.

SUMMARY

1. The ultraviolet absorption and potentiometric titration curves of a number of proteins have been measured. From them the contents of the proteins in tyrosine, tryptophan and amino groups have been estimated.

2. Satisfactorily concordant results have been obtained with normal human serum pseudoglobulin: serum and urinary pseudoglobulin of nephrosis do not differ significantly from normal.

3. Antibodies to types II and III pneumococcus are considered to differ in their properties from normal globulins.

4. Measurements have been made on purified serum albumins: serum and urinary albumin of nephrosis are significantly different from normal.

5. Seroglycoid resembles globulin rather than albumin in its properties.

The authors thank members of the laboratory staff for gifts of blood; Dr L. F. Hewitt for a gift of crystalbumin and of seroglycoid prepared by him; Prof. J. R. Marrack for preparation of the antibody solutions; and Dr Wolff for a specimen of crystallized albumin.

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CLV. THE INFLUENCE OF DIFFERENT CASEIN PREPARATIONS IN RIBOFLAVIN-DEFICIENT DIETS UPON THE APPEARANCE OF CATARACT[1]

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Our experiments showing that nutritional cataract in rats can be prevented by riboflavin [Day *et al.* 1937] and in its early stages can be arrested by riboflavin [Day *et al.* 1938] would seem to solve the problem of the aetiology of this experimental condition. Although there is now no doubt that cataract can readily be produced by riboflavin deficiency under suitable conditions, there appear still to be unanswered questions regarding the exact conditions that must be met to produce this experimental state. By following our experimental procedures O'Brien [1932] and Yudkin [1933] were able to produce cataract in a high percentage of cases. On the other hand, Bourne & Pyke [1935] observed a maximum incidence of only 31 % cataract in their groups of rats. Without giving the details of all his experiments, György [1935] stated: "In an extensive series of experiments in which over 500 rats were involved, we never observed this phenomenon (cataract), either with complete or partial deficiency of the vitamin B_2 complex or with separate deficiencies of lactoflavin or vitamin B_6."

Several years ago we observed that altering the type of casein preparation in our experimental diet caused a noticeable change in the time of appearance of cataract. We therefore obtained samples of casein from different sources, and used them in riboflavin-deficient diets. Our results are interesting in relation to the problem of cataract production by riboflavin deficiency. They may also prove of interest to workers in other fields of nutrition, since casein is a common constituent of experimental diets.

Experimental

All the animals used in these experiments were reared under standardized conditions in our own laboratory. The stock diet used was Sherman's diet 13 [Sherman & Campbell, 1924], which is composed of one-third whole milk powder, two-thirds ground whole wheat and NaCl. This diet was fed *ad lib.* and was supplemented twice a week with fresh lettuce and beef heart. The young were placed on the experimental diet at 21 days of age, and only litters containing 8 or more young were used. Weekly ophthalmoscopic examinations were made and the animals were kept until death.

The experimental riboflavin-deficient diet (No. 625) had the following composition: casein, 18 %; cod liver oil, 2 %; butter fat, 8 %; salt mixture [Osborne & Mendel, 1919], 4 %; and corn (maize) starch, 68 %. The corn starch carried an

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extract of rice polish prepared by the procedure described by Day & Langston [1934]. All the animals in this series received diets of this composition, differing only in the source and treatment of the casein. Several casein preparations were used which are described in the following paragraphs.

I. *Alcohol-extracted.* "Casein, edible, muriatic" (Casein Manufacturing Co. of America), a crude HCl-precipitated casein, which was washed with 60% alcohol in this laboratory, according to the technique of Sherman & Spohn [1923].

II. *Dilute acid-extracted.* "Casein, edible, muriatic", subjected to prolonged washing with acidulated water, according to the method described by McCollum *et al.* [1922].

III. *Labco.* A "vitamin-free" casein (Casein Manufacturing Co.). The method of manufacture has been described briefly by Supplee *et al.* [1936]. It has been used in a number of experiments in this laboratory [Day & Darby, 1936; Day *et al.* 1937; Day *et al.* 1938].

IV. *Harris.* Described by the manufacturers (Harris Laboratories, New York) as a "vitamin-free" casein. The method of preparation is unknown to us.

V. *Glaxo AB.* Glaxo caseinogen AB (Glaxo Laboratories, Ltd., batch F46), described by the manufacturer as "a precipitated acid-washed heat-treated ashless casein". It has been widely used, presumably without further treatment, in many experiments upon the components of the vitamin B complex [Birch *et al.* 1935; György, 1935; Harris, 1935; Prunty & Roscoe, 1935; Ray *et al.* 1935].

VI. *Glaxo ABE.* Glaxo caseinogen ABE (batch F45/E) which the manufacturers state has replaced the Glaxo AB preparation for vitamin A and B experiments. It was described as "heat-treated 'ashless' casein, alcohol-extracted after heat treatment to the maximum possible removal of ether-soluble matter".

VII. *Glaxo ABE—alcohol-extracted.* Glaxo caseinogen ABE subjected to treatment with 60% alcohol in this laboratory, following the method of Sherman & Spohn [1923].

Results and Discussion

Table I gives a summary of our data and Fig. 1 represents a part of the same data in graphic form. The acid-extracted and Harris caseins gave rates of growth, periods of survival, and times for the appearance of cataract almost identical with those given by the Labco casein, as may be seen from a study of the table. For this reason, and also since superimposed lines on the chart would have been confusing, the curves for these two groups were omitted from the chart.

There are 5 obvious criteria that may be used in a study of these data for comparing the 7 casein preparations: (1) average maximum gain, (2) average time to reach maximum weight, (3) average time of appearance of cataract, (4) incidence of cataract and (5) average survival period. Judged by all 5 criteria, the acid-extracted, Labco, and Harris caseins gave almost identical results. It is simplest to assume that these 3 caseins were essentially riboflavin-free, although an alternate explanation is that they all contained the same small quantity of riboflavin.

On the basis of 4 out of the 5 criteria the alcohol-extracted casein appeared to contain a small but significant amount of riboflavin. Although the numbers of animals are not great enough for the data to be treated statistically, we believe that there is significance in the longer survival, delayed appearance of cataract and more prolonged growth in animals receiving this casein; many of the animals receiving the alcohol-extracted and Labco casein diets were littermates, carefully matched as to size and sex.

The two Glaxo casein diets gave much greater growth, the growth was more prolonged, the average survival period was significantly greater and the

Table I. *Summary of data regarding growth, survival and incidence and time of appearance of cataract in groups of rats receiving riboflavin-deficient diets containing different casein preparations*

	No. of rats	No. showing cataract	Av. wt. at start g.	Av. maximum gain g.	Av. no. days to reach max. wt.	Av. no. days for appearance of cataract	Av. no. days survival
I. Alcohol-extracted	15	13	41·1	17	37	83	119
II. Acid-extracted	9	8	39·9	20	28	56	81
III. Labco	17	12	39·6	16	33	50	90
IV. Harris	9	8	37·6	16	23	52	83
V. Glaxo AB	10	7	38·5	42	65	113	133
VI. Glaxo ABE	14	6	38·1	40	62	86	117
VII. Glaxo ABE extracted	5	2	35·8	22	43	75	101

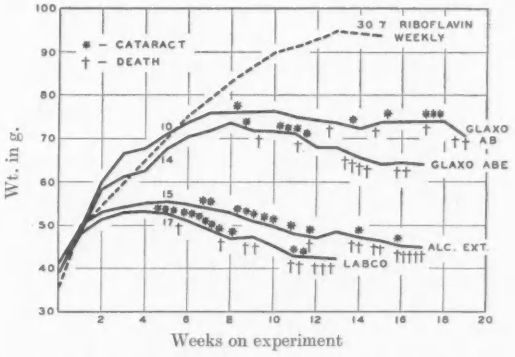

Fig. 1. Composite weight curves of groups of rats which received riboflavin-deficient diets identical in all respects except for the caseins that they contained. The numbers on the curves indicate the number of rats in each group. The asterisks mark the time of appearance of cataract in individual animals, and the daggers mark the death of individual animals. Each curve was terminated at the point of average survival for that group; consequently, animals showing cataract and those dying after that time are not indicated on the chart. The dotted line indicates the average growth of a group of rats receiving the Labco casein diet supplemented with 30γ riboflavin weekly; these animals were killed at the end of 15 weeks.

appearance of cataract was delayed. Furthermore, the percentage incidence of cataract was lower among rats receiving these casein preparations. Whereas the alcohol-extracted casein gave an incidence of 87 %, the Labco 71 %, the Harris 89 % and the acid-extracted 89 % of cataracts, the group of rats receiving the Glaxo AB casein showed an incidence of 70 % and the Glaxo ABE animals only 43 % of cataracts. The incidence for the combined groups receiving Glaxo AB and ABE was 54 %. A consideration of the incidences of cataract for the first 10 weeks of experiment only is even more revealing. They were as follows: alcohol-extracted, 47 %; acid-extracted, 89 %; Labco, 59 %; Harris, 89 %; Glaxo AB, 10 %; and Glaxo ABE, 14 %. Thus, judged by all 5 criteria, the two Glaxo caseins appeared to contain significant amounts of riboflavin.

The seventh sample of casein used—designated in the table as Glaxo ABE extracted—was some of the Glaxo ABE casein subjected to extraction with 60 % alcohol in this laboratory, by the method employed in preparing the alcohol-extracted casein. Unfortunately, there was sufficient material to feed only 5 rats and this experiment was run after most of the animals in the other groups had

died, so this series was not well controlled. However, it is evident that growth and survival were approximately the same as in the group receiving the alcohol-extracted casein.

This evidence of a biological nature indicating the presence of riboflavin in two of the casein samples was confirmed by physical means. Small samples of the caseins were shaken with distilled water and the clear eluates examined under ultraviolet light in a dark room. The source of light was an "Eveready Fluoray" lamp, which emits an intense ultraviolet radiation practically free from visible light. Dilute solutions of riboflavin give a bright greenish fluorescence when placed in this "black light" [Supplee *et al.* 1936]. Eluates from the alcohol-extracted, acid-extracted and Harris caseins gave no fluorescence. An eluate from the Labco casein gave a bluish fluorescence, quite different in colour from that due to riboflavin. However, eluates from the two Glaxo caseins emitted a marked greenish fluorescence, similar to the fluorescence of a pure riboflavin solution, indicating the probable presence of this substance.

Complete riboflavin deficiency results in a pathological picture in the young rat rather different from that seen in partial riboflavin deficiency. The rats receiving acid-extracted, Labco and Harris caseins developed an unkempt appearance of the hair and some alopecia, but extensive alopecia was uncommon and marked involvement of the skin was rare. As shown above, cataract (and also keratitis) was seen in nearly all the animals. This is a typical picture of what we believe to be complete riboflavin deficiency in young rats. On the other hand, animals receiving a small amount of riboflavin—e.g. 30γ a week—show an extensive loss of hair; the body and head frequently become quite denuded. In many animals dry eschars form on various parts of the body, particularly on the face and head. Excoriation of the face and severe ophthalmia are common. Severe keratitis is found in all such animals, but cataract is less common. This latter is the picture that was seen in the animals receiving the Glaxo caseins.

The data presented in this report would seem to indicate that the failure of some investigators to observe cataract in riboflavin deficiency may have been due to the contamination of their caseins with riboflavin. This is probably not the whole explanation of such failure, however. It is probable that the size of animals, previous dietary history, and other conditions also influence the susceptibility of rats to cataract in riboflavin deficiency. Some of these factors are now being studied.

Summary

Seven casein preparations were used as sources of protein in riboflavin-deficient diets. The responses of groups of rats fed these diets were compared by 5 criteria: average maximum growth, average time required to reach maximum weight, average time of appearance of cataract, incidence of cataract and average period of survival. Two commercial "vitamin-free" caseins and a crude casein washed with acidulated water gave nearly identical responses as judged by these 5 criteria. A crude casein washed with dilute alcohol gave slightly longer growth and survival, and the appearance of cataract was somewhat delayed. It is believed that washing with dilute alcohol failed to remove the last trace of riboflavin. Two commercial purified caseins that have been widely used in experiments upon the B-vitamins promoted much greater and longer continued growth, and permitted longer survival. The groups of rats receiving these two casein diets exhibited lower incidences of cataract, and the appearance of cataract was definitely delayed. Thus, judged by all 5 criteria, these two casein preparations appeared to contain significant amounts of riboflavin. Furthermore, eluates

from these two caseins exhibited a marked greenish fluorescence, similar to that produced by riboflavin, when examined in a dark room under ultraviolet light. It is believed that the failure of some investigators to observe cataract in riboflavin deficiency may have been due, in part, to the contamination of their caseins with riboflavin.

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CLVI. THE GONADOTROPIC HORMONE AND THE LEVEL OF BLOOD PHOSPHORUS IN THE HEN

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(*Received 9 May 1938*)

THE presence of phosphoprotein in the blood of laying hens has been demonstrated simultaneously and independently by Roepke & Hughes [1935] and Laskowski [1935, 1]. The phosphoprotein was later isolated in a relatively pure state by Laskowski [1935, 2] and the name serum-vitellin was suggested for it. Roepke & Bushnell [1936] examined its serological properties, and found it to be closely related to ovo-vitellin. Laskowski [1936] investigated the blood of several other species of vertebrates (excluding mammals) and found serum-vitellin during or immediately before the spawning season, in practically all cases.

The object of the present investigation was to examine the influence of gonadotropic hormones on the appearance of serum-vitellin in the blood stream of the hen. That the gonadotropic hormone might be a possible factor in the formation of serum-vitellin was suggested by the work of Bates *et al.* [1935], who found that administration of gonadotropic hormone during 6–16 days increased the true ovarian tissue of resting ovaries by nearly 150 %, and that of active ovaries by 125 %, whilst the weight of resting whole ovaries including yolk was increased by approximately 1000 %; although treatment with gonadotropic hormone does not induce, but on the contrary, inhibits laying. Riddle & Dotti [1936] showed that the administration of gonadotropic hormone results in an increase of serum calcium (which varies in proportion with serum-vitellin during normal laying) in normal, hypophysectomized or thyroidectomized pigeons, but not in castrates.

EXPERIMENTAL

Methods and material. Fully grown Leghorn, Rhode Island Red and Sussex hens were taken for the experiments. The birds were not of uniform age, some of them being in their first, and others in their second laying seasons, and finally, part were in the winter break. The hens were on an all-mash diet, given *ad lib.* Administration of hormone and blood sampling were performed without reference to times of feeding, in view of our previous finding [1934] that the level of phosphorus compounds in plasma is unaffected by feeding.

The injections (usually single) of hormones were made into the pectoral muscle, in the morning. Blood (2–3 ml.) was taken from the wing vein, usually every 48 hr. during 10–12 days following the injection. Total P (P_t) was determined in 0·1–0·2 ml. of citrated plasma, and the individual P fractions, acid-soluble (P_{in}), lipin (P_l) and protein P (P_p) were determined in the same 0·5–1·0 ml. sample by the procedure previously described [Laskowski, 1935, 1]. In those experiments in which only P_t was determined the presence of serum-vitellin was sought qualitatively, by tenfold dilution of the plasma with water.

Phosphorus was determined by Martland & Robison's modification [1926] of Briggs' method [1922]. Most of the hormone preparations used were commercial

samples; in some cases preparations were made according to Bates & Riddle [1935] from fresh pituitaries, and one of these preparations was further purified according to Cartland & Nelson [1937].

Results

1. Antex Leo (*Lovens Kemiske Fabrik, Copenhagen*). Fig. 1 shows the results of single injections of 30 mg. of Antex (240 mouse units), into two non-laying hens. In both cases P_t rose sharply, to a maximum of 140 mg. per 100 ml. plasma on the 6th day, an increase of about 1000 %. According to our previous findings

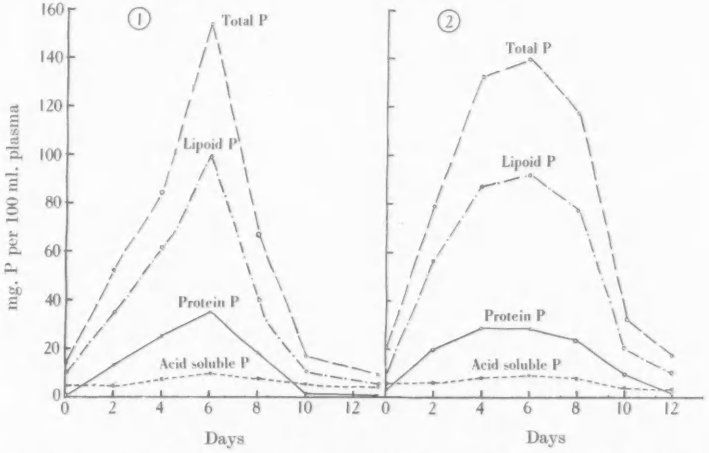

Fig. 1. Effect of a single injection of 30 mg. Antex (240 mouse units) on the individual fractions of plasma P in non-laying hens. Curves 1. Brown Leghorn No. 11, 2nd year, winter break in laying began 5 weeks before injection. Curves 2. Rhode Island Red No. 10, 2nd year, exhibited partial broodiness since laying of last egg 3 days before injection.

[Laskowski, 1935, 1], the P_t content of the plasma of non-laying hens varies between 8 and 20 mg., and of laying hens between 18 and 60 mg. per 100 ml.; the highest values now obtained are thus about three times higher than the maximum physiological value. The plasma becomes turbid and strongly opalescent following the injection. All the P fractions participate in the effect, though in different degrees. Thus P_{in} (practically identical with inorganic P in plasma) changes least, its content being only doubled. The bulk of the rise in P_t is due to P_l, which attains a value of 90 mg. per 100 ml. The greatest relative increase is shown by P_p, only traces of which are found in non-laying hens, while after Antex injections it amounts to over 30 mg. per 100 ml., corresponding with a serum-vitellin content of 2·7 %. The curves are symmetrical, return to normal being attained after 10–12 days. Eggs were not laid during this period.

It appears probable that the appearance of serum-vitellin under physiological conditions (during laying) is also due to gonadotropic hormone, since the changes in the individual P fractions are of the same order, although of different magnitudes. The chief difference appears to be that normally the serum-vitellin goes to form the developing ova, which is not possible in the conditions of the present experiments.

The response of different individuals to identical doses is very variable, and this applies both to the maximum increments in P and to the time required to attain this maximum. Curves 1–2, Fig. 1, and 3–6, Fig. 2, represent changes in P_t after injection of 30 mg. of Antex into different birds. A comparison of curves 5 and 6 with the remaining ones shows that the curves are less symmetrical in the case of laying than in that of non-laying hens, as a result of the higher initial values in the former case. Curve 3, in which the injection was given on 3 successive days instead of in one dose, differs from curve 4 chiefly in that the peak is attained later; however in other cases of single injections, maximum P_t was also found on the 6th day.

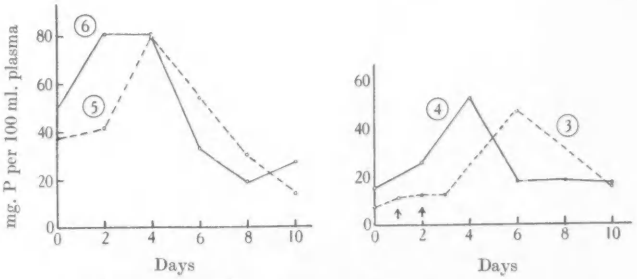

Fig. 2. Variability of response to the same dose (30 mg.) of Antex in different hens (curves 3 and 4 non-laying, 5 and 6 laying). Curve 3. Brown Leghorn No. 6, 1st year, has not yet laid. 3 daily 10 mg. doses. Curve 4. Rhode Island Red No. 16, 2nd year, winter break in laying began 6 weeks before single injection. Curve 5. White Leghorn No. 15, 2nd year, laid an egg on the day of injection. Curve 6. Rhode Island Red No. 27, 2nd year, laid an egg on the day preceding, and a second egg on the second day after injection.

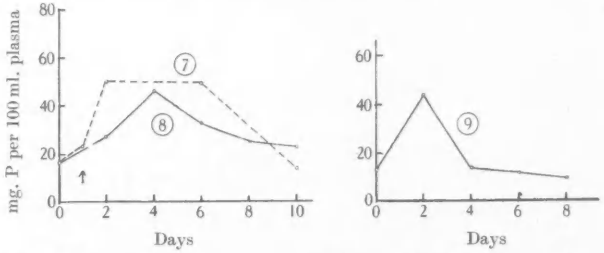

Fig. 3. Effect of different doses of Antex on the P_t of non-laying hens. Curve 7. Brown Leghorn No. 7, 1st year, has not yet laid. One injection of 50 mg. Curve 8. Rhode Island Red No. 8, 2nd year, winter break in laying began 8 weeks before injection. Three successive daily injections of 15 mg. each. Curve 9. Rhode Island Red No. 6, 2nd year, winter break in laying began 2 weeks before injection of a single 15 mg. dose.

In view of this individual variability of response no close correlation between dose and response could be expected. Curves 7–9, Fig. 3, in conjunction with the curves of Figs. 1 and 2 illustrate this point.

These differences in response may probably be ascribed to the variable physiological condition of the bird. Some light is thrown on this point by the experiments represented in Fig. 4, in which the injections were repeated after the effects of the first dose were no longer apparent. It is evident that the birds were still refractory to the action of Antex at this stage. In two other experiments a

positive response was obtained on the 17th day after the first injection in one case, and a negative response in the other.

In view of the lack of uniformity of response, and of the large amounts of hormone needed, it seems improbable that the effect in question could be applied to the assay of gonadotropic hormone.

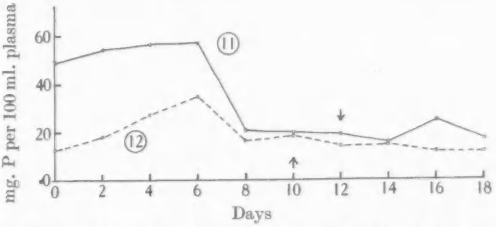

Fig. 4. Effect of repeated injection of the same (30 mg.) dose of Antex into the same hen. Curve 11. Sussex No. 16, 2nd year, winter break in laying commenced 8 weeks before the first injection, and the first egg of the new season was laid on the day preceding injection. Curve 12. White Leghorn No. 10, 2nd year, winter break in laying began 8 weeks before injection.

2. Other gonadotropic hormone preparations. Reiss *et al.* [1933] showed that the growth of the testes and comb of young cockerels is accelerated by pituitary and serum hormones but not by those of urinary origin, and this finding was confirmed by Hamburger [1934] and Pfaltz [1936]. It was concluded therefrom that birds are insensitive to urinary hormones, and experiments were therefore instituted with the object of testing the validity of this conclusion, with regard to blood P.

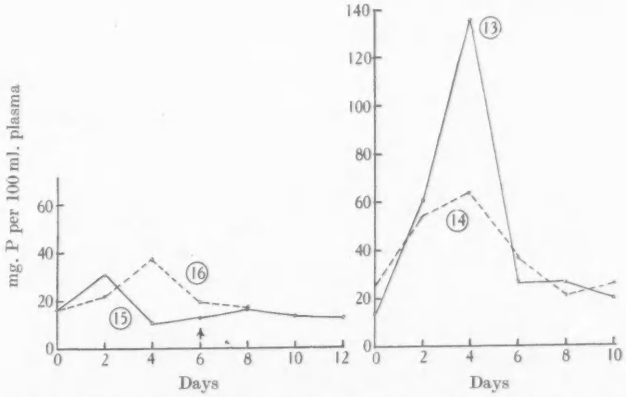

Fig. 5. Action of pituitary hormones. Curve 13. White Leghorn No. 3, 2nd year, winter break in laying commenced 1 week before injection. 0·5 g. of anterior lobe pulp and 30 mg. of Antex injected simultaneously. Curve 14. Rhode Island Red No. 12, 2nd year, laying well. 30 mg. of Antex and 100 mg. of Allen and Hanbury's prolactin injected. Five eggs were laid during the 5 days following, after which laying ceased. Curve 15. Rhode Island Red No. 11, 2nd year, winter break in laying set in 6 weeks before injection of 150 mg. of an anterior lobe preparation made according to Bates & Riddle. The injection was repeated after 6 days. Curve 16. Rhode Island Red No. 10, 2nd year, winter break in laying set in 6 weeks before injection of 52 mg. (equivalent to 30 fresh pituitaries) of purified anterior lobe preparation.

In our first experiments injections of 0·5–0·8 g. of fresh ox pituitary anterior lobe pulp produced no effect, whilst higher doses were lethal to the birds. This

negative result might have been due to presence of inhibitory or antagonistic factors in the pulp; this possibility was tested by experiments in which 30 mg. of Antex and 0·5 g. of anterior lobe pulp or 100 mg. (180 crop-sac units) of Allen and Hanbury's prolactin were injected simultaneously (curves 13 and 14, Fig. 5). The results were positive in both cases and afford no support to the hypothesis of interference by antagonistic substances. It is of interest that the hen injected with prolactin did not cease laying immediately, but only on the 6th day; in all other cases Antex caused immediate cessation of laying.

In the next experiment 150 mg. of an anterior lobe preparation, equivalent to 20 fresh ox pituitaries, made by the method of Bates & Riddle [1935], were injected. The effect (curve 15) was definite, though small. A second injection on the 6th day had no effect. A purer preparation (52 mg., equivalent to 30 pituitaries) also gave a slight, but definite effect (curve 16). Positive effects were also obtained with an injection of 300 rat units of a mare-serum gonadotropic hormone (Dr Roussel, Paris).

All urinary preparations tested (300–500 rat units of Schering-Kahlbaum, Organon, Hoffman-La Roche, Richter, Klawe gonadotropic hormones) were inactive when injected into non-laying or laying hens; they did not affect blood P, nor did they interrupt laying.

Summary

1. After a single suitable dose of gonadotropic hormone (pituitary or mare-serum) the appearance of serum-vitellin in the blood stream of resting hens was observed. The other P fractions of the plasma rose simultaneously. In some cases a value for total P of 140 mg. per 100 ml. was found, being ten times higher than the normal value for resting hens and three times higher than for laying hens.

2. Gonadotropic hormones of urinary origin do not influence blood P.

3. The effect cannot be applied to assay of the hormones, but may serve for the differentiation of urinary and pituitary hormones.

This work was partly carried out at the Lister Institute during the tenure of a Rockefeller Foundation Fellowship, for which I express my indebtedness. My thanks are also due to the Polish National Culture Fund for financial support, and to Drs G. A. Grant, M. Guggenheim and R. Truszkowski for gifts of hormone preparations.

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CLVII. THE NITROGEN PARTITION IN BLOOD CLOTTING

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(*Received 30 May 1938*)

WHILE there has been considerable controversy for many years as to the enzyme nature of thrombin, the weight of evidence, as summarized by Eagle [1935] recently, is in favour of thrombin being an enzyme. Little is known, however, regarding the nature of the reaction catalysed. Numerous workers have put forward the view that thrombin is a proteolytic enzyme similar to trypsin. This assumption was based on the fact that, under certain conditions, trypsin clots blood and plasma but the finding of Dale & Walpole [1916] that trypsin has an indirect action on the clotting system suggests other possible explanations. As a result of the supposed proteolytic action, a number of workers have assumed that fibrin is not the sole product of the reaction. Such a possibility can be tested by studying the nitrogen partition during clotting, provided that the fibrinogen is free from other protein and a sufficiently active thrombin is used. While early workers in the subject [*v.* Hsu & Wu, 1933] attempted studies of this nature, their results are vitiated by the use of impure fibrinogen. Hsu & Wu used much purer preparations and found only 6·7 % of the fibrinogen N in the serum after clotting, 93 % being recovered as fibrin. The significance of this 7 % difference has yet to be established.

MATERIALS

Fibrinogen. This was prepared by the method of Florkin [1930] which involves three precipitations of the protein with NaCl. By maintaining a *p*H of 6·0 and conducting all operations in the cold room at 2°, the method yields a chemical individual of constant and reproducible solubility, which can be kept for some weeks without change in solubility. Before use, the fibrinogen was precipitated with a half volume of 3*M* potassium phosphate buffer of *p*H 6·6 and redissolved in *M*/4 phosphate buffer containing toluene to give a concentration of 0·5 mg. N per ml.

Thrombin. We are indebted to Mr R. A. Mustard for a large amount of thrombin, prepared by the method of Mellanby [1930, 1933]. A solution was made from the dry powder and impurities precipitated by adjusting to *p*H 5·3. The solution was then brought to neutrality and toluene added. For use, it was diluted with distilled water.

METHODS

The reaction. The reaction was carried out in pyrex test tubes, 12 × 1·4 cm.; 2 ml. of fibrinogen were placed in the tube, 3 ml. of water added and the tube placed in a water bath at 25°. At zero time, 1 ml. of thrombin solution was added, the tube stoppered with a paraffined cork, inverted to mix the contents and then placed in a rocker in the bath. The tube was completely immersed and was rocked gently so that the contents flowed from one end of the tube to the other about 30 times a minute. This served both to keep the solution homogeneously mixed and also to break up the fibrin clot as it formed.

The reaction was stopped by centrifuging the tube in a small angle centrifuge for 4 min. 2 min. after beginning centrifuging was arbitrarily taken as the end of the reaction time. The supernatant solution was decanted off and the fibrin washed with 2 ml. of 1 % saline, any fibrin adhering to the cork being removed with a probe and added to the saline. The tube was again centrifuged and the wash fluid added to the supernatant. N determinations were then made on the fibrin and the supernatant, the digestion of the fibrin being carried out in the same test tube as the reaction. The nitrogen was determined by the micro-Kjeldahl method using an apparatus of the improved Parnas-Wagner type. $N/140$ HCl and $N/140$ NaOH were used for absorption and titration respectively.

The nitrogen partition during clotting. As Hsu & Wu have pointed out, it is necessary to study this over the whole reaction and not just at equilibrium. This procedure was followed in all the experiments and Fig. 1 shows a curve typical of

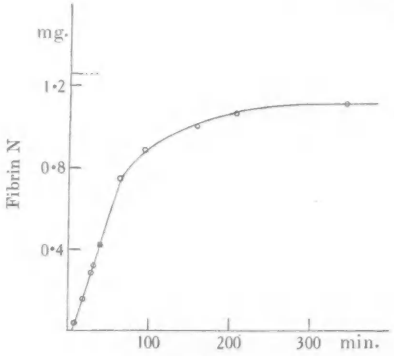

Fig. 1.

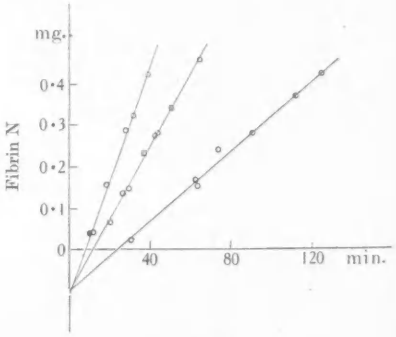

Fig. 2.

Fig. 1. Exp. 346. Fibrinogen N, 1·255 mg.; thrombin N, 0·007 mg. Phosphate $M/8$. Total volume, 6 ml.

Fig. 2. *a*, Fibrinogen No. 43, thrombin No. 46 (0·007 mg. *N*). *b* + *c*, Fibrinogen No. 55, thrombin No. 70. In *b* thrombin N was 0·0028 mg.; in *c*, 0·0014 mg. Total fibrinogen N was 1·09 mg. Equations of the lines were found to be: (*a*) F(fibrin nitrogen) $= 0{\cdot}01344t - 0{\cdot}1078$; (*b*) $= 0{\cdot}00876t - 0{\cdot}1038$; (*c*) $= 0{\cdot}00416t - 0{\cdot}0953$. Average value of $a = 0{\cdot}1021$ mg. N $= 0{\cdot}0170$ mg. N/ml.

those obtained. Two points are to be observed with regard to this curve—(1) not all the fibrinogen N is recovered as fibrin N, $1{\cdot}255 - 1{\cdot}125 = 0{\cdot}130$ mg. N remaining in the supernatant, and (2) a small lag phase is present. Before assuming that the N in the serum represents another product of the reaction it is necessary to investigate the possibility that it is due to the solubility of fibrin, and the lag phase provides a means of testing this hypothesis. If fibrin has a measurable solubility, a measurable time will elapse at the beginning of the reaction before the solution is saturated with fibrin and the fibrin begins to precipitate. Therefore, by continuing the initial linear portion of the curve below the time axis, it should cut the fibrin axis at the value representing the dissolved fibrin. This has been done in Fig. 2 for various concentrations of thrombin and it can be seen that the intercept on the fibrin axis is 0·10 mg. in each case. The close agreement observed suggests that the lag phase is simply due to the solubility of fibrin. Hence, it is probable that the difference between the fibrinogen and fibrin nitrogen is also due to the solubility of the fibrin, so that,

if any N is split off in the conversion, it is less than 0·5 % of the original N. The value reported represents a solubility of 0·0170 mg. N per ml. or 0·102 g. protein per litre. Hsu & Wu's values when converted to the same basis show close agreement, 0·017 ± 0·006 mg. per ml.[1]

It can be observed that when the progress curve is drawn to allow for the solubility of the fibrin, the curve is that typical of many enzyme reactions. This is in contrast to the results of Klinke & Elias [1931] and Kugelmass [1925] who found the reaction to be autocatalytic. The difference may be due to the different physical conditions used.

Fibrinolytic action of purified thrombin. A number of reports have appeared in the literature suggesting that thrombin has a fibrinolytic action (see Hirose). The most conclusive is that of Hirose [1934]. She reported that using sterile thrombin and fibrinogen, fibrinolysis occurred in 3 to 6 days. To our surprise, we found that our purified thrombin had a very high fibrinolytic action under the conditions of our experiments, measurable fibrinolysis occurring 4 hr. after the addition of thrombin. Fig. 3 shows an experiment of this nature. The fibrin formed reached a maximum in 3 hr. and then rapidly decreased. Increasing the thrombin concentration decreased the total amount of fibrin formed and increased the rate of fibrinolysis. Controls showed that it was not due to the shaking, and that removal of the supernatant containing most of the thrombin at the end of clotting resulted in the fibrinolysis being decreased proportionately. That such a marked action of thrombin has not been observed before may be due to the use of less purified reagents. As found by Hirose, the product of the reaction appears to be Hammarsten's fibrinoglobulin, no non-protein nitrogen appearing during the reaction.

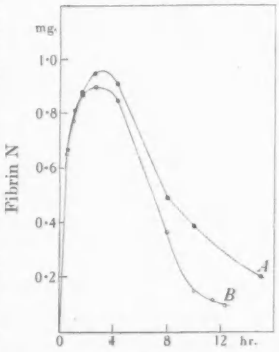

Fig. 3. Fibrinogen N = 1·09 mg. Thrombin N in *A*, 0·064 mg.; in *B*, 0·128 mg.

It is difficult to prove that the fibrinolytic enzyme is actually thrombin and not another enzyme which has accompanied it through the purification process. As Mellanby's preparation of thrombin involves the preparation of prothrombin followed by its spontaneous activation and the removal of impurities which do not undergo the same change in properties, it seems unlikely that the fibrinolysis is due to an accompanying enzyme.

This work was originally undertaken with a view to applying the method (determination of the fibrin nitrogen by micro-Kjeldahl analysis) as a quantitative basis for the study of various problems in blood clotting. This has not proved feasible chiefly because of the difficulty in stopping the reaction to take samples. Any other method than centrifuging results either in the precipitation of the fibrinogen or the re-solution of the fibrin.

[1] A further indirect proof of the solubility hypothesis is the solubility of fibrinogen in salts used as precipitants. Campbell & Hanna [1937] have recently shown that fibrinogen is precipitated with 12·5% sodium sulphite so as to yield values which check with the Van Slyke & Cullen method of estimating fibrin. In a series of experiments the solubility of fibrinogen under the conditions of their method was found to be 0·043 mg. N per ml., the equivalent of 0·014 mg. fibrin N per ml. in the Van Slyke & Cullen method.

SUMMARY

When thrombin acts on a solution of fibrinogen, free from other proteins, all the fibrinogen N appears as fibrin nitrogen. Owing to the solubility of fibrin, however, as much as 10% of the fibrin nitrogen may be present in the supernatant. Purified thrombin preparations have a marked fibrinolytic action.

Note added 5 July, 1938. Since this paper was sent to press the results of a similar study by Presnell (*Amer. J. Physiol.* **122**, 596, 1938) have been published. When his results are analysed, they indicate an interpretation similar to that given above, namely that the difference in the fibrinogen nitrogen is due to the solubility of fibrin rather than to a "proteolytic" action of thrombin.

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CLVIII. ENZYME SYSTEM OF *BACT. SUBOXYDANS*

II. EFFECT OF ACIDS AND *p*H

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(*Received 20 May 1938*)

It has been shown [Butlin, 1936, 1938] that the addition of chalk to maize wort agar used as growth medium for *Bact. suboxydans* has a striking effect on that organism's enzymic activity. This effect was ascribed to the neutralization by the chalk of the acid formed by the growing culture on the medium. To obtain further evidence for this, the direct action of acids on the highly oxidizing suspension derived from chalk-containing maize wort agar was examined.

Experimental

A suspension of *Bact. suboxydans* (dry weight 25 mg./ml.) was prepared as previously described [Butlin, 1936], after 2 days' growth at 30° on maize wort agar containing 2% chalk. 4 ml. portions of the main suspension were treated with 1 ml. acid of various strengths, left for ½ hr., centrifuged and washed twice with sterile saline, and finally made up with 6 ml. sterile saline. The control received the same treatment except that water was substituted for acid. The O_2 uptake and CO_2 production with 0·3 ml. 0·1 *M* glucose were then determined in the Barcroft differential manometer by the usual methods, phosphate buffers of *p*H 6·0 and a temperature of 30° being used throughout.

Figs. 1 and 2 contain the O_2 uptake-time and CO_2 evolution-time curves with glucose as substrate given by suspensions treated with varying concentrations of HCl. The latter are expressed in terms of the actual strength of acid in contact with the bacterial cells during treatment. It should be emphasized that after treatment the cells were well washed and their action with glucose conducted at *p*H 6·0.

The results obtained bear a strong resemblance to those showing the effect of time of growth of the culture on the medium [Butlin, 1938], except that there was no initial increase in activity. The control suspension gave the normal behaviour of *Bact. suboxydans* with glucose at *p*H 6·0, i.e. rapid initial O_2 uptake, relatively slow initial evolution of CO_2, followed by an increase in the latter until its rate of evolution became equal to the rate of O_2 uptake. Treatment of the suspension with 0·001 *N* HCl had only slight effect, but with increasing concentration of acid the O_2 uptake and evolution of CO_2 decreased by approximately equal amounts until the latter entirely ceased, when the O_2 uptake (470 μl.) was equivalent to slightly less than 1½ atoms O for each molecule of glucose. This condition, in which the reaction with glucose was reduced to a simple oxidation process, was attained by treatment of the suspension with 0·006 *N* HCl. From this stage the effect of treatment with more acid was less intense and a concentration as great as 0·02 *N* was required to reduce the O_2 uptake to the equivalent of

1 atom O (344μl.). Despite the large decrease in total O_2 uptake and the total inhibition of CO_2 production, it is clear from Fig. 1 that the initial rate of O_2 absorption, up to about 350μl., is practically unaltered by treatment of the suspension with concentrations of HCl not greater than 0·02N. From this it is

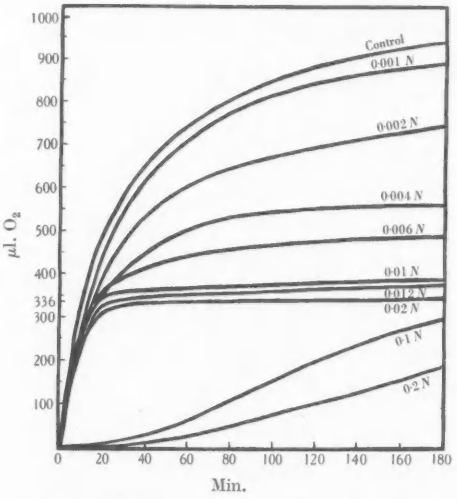

Fig. 1. O_2 uptakes, with 0·3 ml. 0·1 M glucose as substrate, of suspensions of *Bact. suboxydans* treated with different concentrations of HCl and then washed. Temp. 30°. *p*H 6·0.

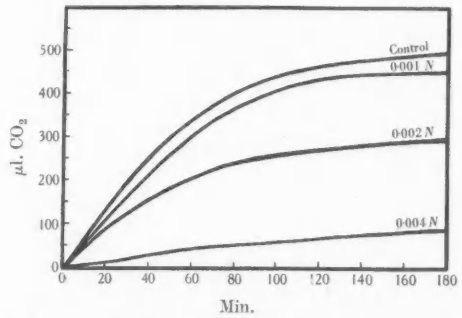

Fig. 2. Evolution of CO_2, with 0·3 ml. 0·1 M glucose as substrate, of suspensions of *Bact. suboxydans* treated with different concentrations of HCl and then washed. Temp. 30°. *p*H 6·0.

inferred that the rapid initial O_2 uptake is chiefly the result of a simple oxidation process. With concentrations of HCl greater than 0·02N the O_2 uptake was gradually inhibited until there was complete aerobic inactivation after treatment with 0·4N acid. The variation with different concentrations of acid of total O_2 uptake and CO_2 production given by the treated suspensions with glucose after 3 hr. in the manometer is shown in Fig. 3. The rapid fall in the curve at the beginning illustrates the relatively great effect of the smaller concentrations of

acid, which decrease O_2 uptake and CO_2 evolution by equal amounts, up to the point when the CO_2 production is completely inhibited (0·006*N* HCl). After this stage there is much greater resistance to the effect of increasing acid concentration, shown by the much flatter portion of the curve at an O_2 uptake equivalent approximately to 1 atom O. The real effect is even greater than is apparent from Fig. 3 owing to the necessity for telescoping the scale for the larger acid concentrations.

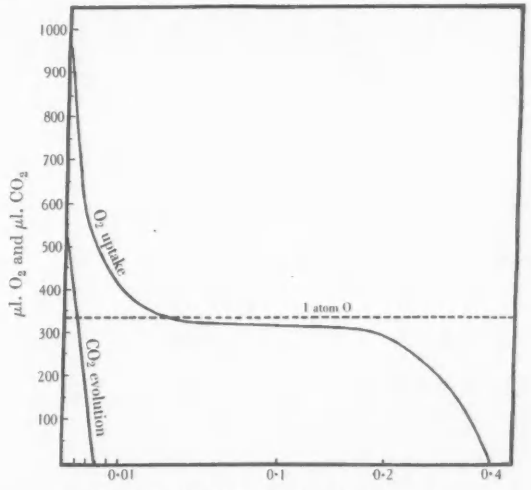

Fig. 3. O_2 uptake and CO_2 evolution, after 3 hr. in manometer with 0·3 ml. 0·1 *M* glucose as substrate, of suspensions of *Bact. suboxydans* treated with different concentrations of HCl and then washed. Temp. 30°. *p*H 6·1.

These results indicate strongly a differential action of acid on the enzymic system of *Bact. suboxydans* and constitute further evidence for the conclusions reached in a previous paper [Butlin, 1938]. There it was suggested that *Bact. suboxydans* possesses an enzyme system containing at least two important components. One, the more sensitive to acid, catalyses a reaction in which the O_2 absorbed is equal to the CO_2 evolved. The rapidly falling portion of the curve in Fig. 3, where the O_2 uptake and CO_2 evolution decrease by equal amounts when the suspension has been treated with comparatively small concentrations of acid, is contributory evidence for the existence of this component. The other component catalyses a simple oxidation of glucose to gluconic acid in which 1 atom O for each molecule of glucose is absorbed and a partial further simple oxidation to 5-ketogluconic acid. This is represented by the flatter portion of the curve, which clearly illustrates its resistance to the action of acid.

These data were obtained with HCl. Gluconic acid behaved in a similar manner.

Influence of p*H*

The above results may be regarded not merely as the result of the action of acid on the enzyme system of *Bact. suboxydans* but also as the consequence of subjecting the suspensions to various *p*H levels for a certain period, the cells

being then washed and their action on glucose determined at *p*H 6·0. Fig. 4 gives the *p*H produced by the addition of increasing concentrations of HCl to the suspensions, which consisted of cells in physiological saline, and to saline alone. The buffering action of the cells is apparent from the figure.

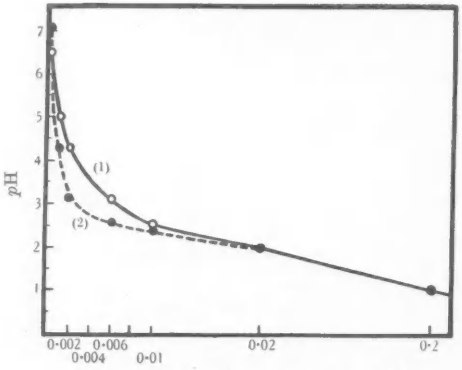

Fig. 4.

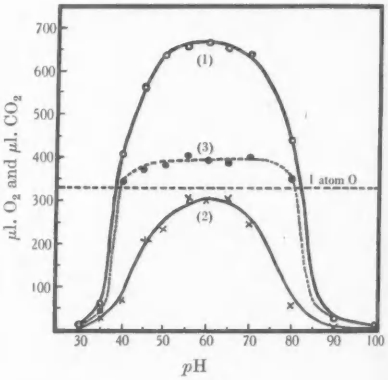

Fig. 5.

Fig. 4. *p*H produced by different concentrations of HCl. (1) o—o On suspensions of *Bact. suboxydans* in saline. (2) •----• on saline alone.

Fig. 5. (1) O_2 uptake o—o; (2) CO_2 evolution ×—× and (3) "O_2 uptake unaccompanied by CO_2 evolution" after 1 hr. in manometer with 0·3 ml. 0·1 *M* glucose as substrate, given by suspensions of *Bact. suboxydans* at different *p*H's.

The results obtained by the action of acids gave evidence for the presence of two components in the enzyme system of *Bact. suboxydans*. Similar conclusions may be drawn from the values for O_2 uptake, CO_2 output and "O_2 uptake unaccompanied by CO_2 output" given with glucose by well washed suspensions of the organism at different *p*H after 1 hr. in the Barcroft manometer (Fig. 5). For *p*H 3·0, 4·0, 5·0 phthalate, for 6·0, 7·0 and 8·0 phosphate and for 9·0 and 10·0 borate buffers were used. A temperature of 30° was used throughout. The results show that, though there is little significant difference in CO_2 production or total O_2 uptake at *p*H 5·0, 6·0 and 7·0, there is a rapid decrease below 5·0 and over 7·0. On the other hand the values for "O_2 uptake unaccompanied by CO_2 output" remain practically constant between *p*H 4·0 and 8·0 at a value just above the equivalent of 1 atom O, whereas at *p*H 4·0 and 8·0 the CO_2 production is practically nil. Here again we have the phenomenon of CO_2 production ceasing when the reaction represented by "O_2 uptake unaccompanied by CO_2 output" is intact. This constitutes further evidence for the suggested two-component enzymic structure of *Bact. suboxydans* outlined above.

Cultures grown on other media

The evolution of CO_2 with glucose is not confined to suspensions derived from cultures grown on maize wort agar with or without chalk. In suitable conditions it has been observed with cultures from every other growth medium so far employed, e.g. glycerol-yeast agar with and without chalk, malt wort agar with and without chalk, bouillon agar with chalk, glycerol-yeast extract solution, 10%

glucose-chalk-yeast extract solution and 10% sorbose-yeast extract solution. This property of *Bact. suboxydans* is therefore not specific to cultures grown on maize wort agar but is a normal characteristic of the organism.

Discussion

Previous experimental results [Butlin, 1938] suggested that *Bact. suboxydans* possesses an enzyme system containing not only a component catalysing a simple oxidation process without production of CO_2 but another component which catalyses a reaction in which the O_2 absorbed is equal to the CO_2 evolved. The work described in the present paper presents confirmatory evidence for this suggestion, based on the differential effect of *p*H and of the direct action of acid on the enzyme system.

In ordinary liquid culture *Bact. suboxydans* is characterized by its slight intensity of oxidation as exemplified in such typical and almost quantitative transformations as glucose to gluconic acid, sorbitol to sorbose and glycerol to dihydroxyacetone. It is therefore surprising that it should possess a highly oxidizing factor in its enzymic equipment as shown by the action of washed suspensions of the organism with glucose under Barcroft conditions. It might be suggested that the strain used in the experiments had altered in character since its isolation. Similar results, however, have been obtained from 5 cultures from different sources. Moreover, these highly oxidizing suspensions when inoculated into liquid cultures show the modified oxidations characteristic of the organism. A more probable explanation is that *Bact. suboxydans* is unable appreciably to exert its more highly oxidizing function because of the conditions it creates in the liquid cultures usually employed. This function, which is sensitive to acid conditions, will be inhibited if acid is formed in sufficient quantity, leaving intact the more moderately oxidizing component. This explanation is readily applicable to the case where glucose is the substrate in suitable liquid medium of initial *p*H 6·0: the rapid formation of gluconic acid by the moderately oxidizing component would quickly inhibit the more drastic oxidation. At first sight it does not appear to explain the almost quantitative production of sorbose from sorbitol and dihydroxyacetone from glycerol, the chief final products being neutral, when suitable concentrations of the substrates in yeast water at *p*H 6·0 are inoculated with suspensions of *Bact. suboxydans*. In practice, however, it is found that owing to the formation of some unknown acid the *p*H of these solutions falls rapidly to 4·5 and often lower, and at these levels only the less actively oxidizing component is able to function. *Bact. suboxydans*, therefore, while possessing a highly oxidizing component in its enzyme system, is usually unable appreciably to utilize it and is chiefly noted for its property of restricted oxidation.

It is possible that the CO_2 production with its accompanying equal O_2 absorption is due to some anabolic process of the nature suggested by Barker [1936], Giesberger [1936] and Clifton [1937], in which the substrate is converted into cell materials simultaneously with the normal oxidative process. This possibility is being investigated.

Summary

From further evidence based on the differential effect of *p*H and of the direct action of acid on the aerobic activity of washed suspensions, it is concluded that *Bact. suboxydans* possesses an enzyme system containing at least two important components. One, less sensitive to acid but more rapid in its action, catalyses a simple oxidation process. The other component catalyses a reaction in which the O_2 absorbed is equal to the CO_2 evolved.

The writer wishes to express his sincere thanks to Dr A. C. Thaysen for his interest and advice, and to Mr W. H. D. Wince for technical assistance. This work was carried out as part of the programme of the Chemistry Research Board and is published with the permission of the Director of Chemical Research in the Department of Scientific and Industrial Research.

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CLIX. CHOLESTEROL METABOLISM

I. ACIDS APPARENTLY CONCERNED IN THE METABOLISM OF CHOLESTEROL

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From the Biochemical Laboratory, Cambridge

(*Received 1 June 1938*)

The question of the origin of cholesterol in animals has been the subject of numerous researches but the problem of the metabolism of cholesterol has not commanded the same attention. Page & Menschick [1932] concluded that approximately 70% of the cholesterol ingested by rabbits could not be accounted for by the sterol excreted in the faeces or by that stored in the animals. Schonheimer & Breusch [1933] found the cholesterol balance in mice to be negative or positive, depending on the supply of exogenous sterol. Beumer & Fasold [1933] showed that the rat also is apparently capable of metabolizing cholesterol. This conclusion was confirmed by Cook [1936, 1937], who found that rats fed a diet containing 2% cholesterol were able to metabolize 20–30% of the amount ingested.

This communication gives the results of further balance experiments. It is to be remarked that this work must be regarded as preliminary in nature and only indicative as to the possible fate of the cholesterol. Experiments on the acidic constituents of the faeces have yielded such consistent results that it is felt that, even if the direct relation of these acidic substances to cholesterol is not yet established, there is little doubt that certain fatty acids are concerned in, or associated with, cholesterol metabolism.

Experimental

The animals used were drawn from the laboratory piebald stock. As far as possible litter-mates were taken but if these were not available animals of approximately the same weights were used. The room in which the animals were kept was maintained at a constant temperature. The animals were kept in Hopkins metabolism cages, the urine and faeces being collected separately. The experiments can be divided into four dietary groups, three of which received different fats, and one no fat. The diets fed to the animals were made up as shown in Table I. In the diets of the control animals the cholesterol was omitted.

The cholesterol used was the specially recrystallized product of Glaxo, "Cholesterol G. L. Puriss. Recryst." The cholesterol was first dissolved in the fat portion of the diet by heating on a water bath, the molten mass being then well mixed with the other constituents.

The data with respect to the duration of the experiments, the weights of the animals and the amounts of food and cholesterol consumed are given in Table II. Exps. 1–4 belong to group A diet, exps. 5–7 to group B, Exps. 8 and 9 to group C and Exps. 10 and 11 to group D. Exps. 8 and 9 were done in collaboration with G. N. Jenkins. The results in Exps. 8 and 9, and Exps. 10 and 11 are the means of 3 animals in each case. In two instances, Exps. 1*a* and 6*a*, the animals died during the course of the experiment, and to counteract this effect as far as possible the

[1] Beit Memorial Research Fellow.

Table I. *Rats' diets*

All values in g.

	Group and type of fat			
	A Arachis oil	*B* Triolein (B.D.H.)	*C* Beef dripping	*D* Fat-free
Caseinogen	23·0	23·0	23·0	23·0
Rice starch	40·0	40·0	40·0	50·0
Cane sugar	17·0	12·0	17·0	22·0
Fat	15·0	20·0	15·0	0·0
Salt mixture	5·0	5·0	5·0	5·0
Yeast	7·5	7·5	7·5	7·5
Cod liver oil	2·5	2·5	2·5	0·0
Radiostoleum	0·0	0·0	0·0	1 drop per diem
Cholesterol (Glaxo)	2·0	2·0	2·0	2·0
Calories per 100 g. food	426·0	448·0	426·0	347·0

Notes. The caseinogen used was "Glaxo ashless casein E"; the salt mixture No. 185 of McCollum & Davis [1915]. The yeast was a dried yeast preparation of trade name "Ceremealo". No calorigenic value is given to the cholesterol.

Table II. *The duration of experiment, animals' weights, food and cholesterol consumptions of the experimental rats. In Exps. 8, 9, 10 and 11 three animals were used in each experiment. The weight figures are mean values.*

			Animals					
				Wt. g.				
Exp. No.	Diet	Days on diet	Sex	Start	End	Gain	Total amount of food g.	Total amount of cholesterol fed g.
1 *a*	15% arachis oil	40*	♂	32	65	33	1618	—
b	controls	123		30	222	192		
2 *a*	Plus cholesterol	98	♂	32	176	144	1574	28·1
b		100		34	192	158		
3 *a*	15% arachis oil	123	♀	32	160	128	2015	—
b	controls			29	137	108		
4 *a*	Plus cholesterol	109	♀	29	116	87	1611	28·7
b				30	121	91		
5 *a*	20% triolein	29	♂	82	145	63	550	—
b	control			93	144	51		
6 *a*	Plus cholesterol	22*	♂	86	76	−10	560	10·0
b		47		95	157	62		
7 *a*	Plus cholesterol	34	♂	44	97	53	503	9·0
b				39	92	53		
8	15% beef dripping controls	22	♀	193	187	− 6	715	—
9	Plus cholesterol	22	♀	212	231	19	818	14·6
10	Fat free diet	21	♀	104	117	13	645	—
11	Plus cholesterol	21	♀	97	133	36	657	12·0

* Animal died.

remaining animals were allowed to continue for a longer time so that the food intake was approximately the same as for the other members of the series. The results for growth and food intake are in good agreement with the previously published figures.

The animals were bled under anaesthesia, the blood being analysed with the carcasses. The livers were removed from the well-bled animal and weighed. The values given for liver as % body weight are the weights of the blood-free livers as % of the gross weights of the animals just before death. The animals were gutted, the gut and contents being analysed together. This material was kept free from mesenteric fat as far as possible. The remaining portions were analysed as carcass. In some cases the livers were analysed individually.

Methods of analysis. A revision has been made in the methods of analysis. It was found that the Leathes & Raper method tended to give low and inconsistent results. The method adopted is modelled on that of Kumagawa & Suto [1908]. A prolonged alkaline hydrolysis is necessary, and the acidified mass must be extracted at least thrice with ether. The crude lipoid was then further saponified and the unsaponifiable matter extracted. The alkaline portion was then acidified and extracted with light petroleum, the acids being weighed. The lipoid in the case of the livers is the sum of the values obtained for the unsaponifiable matter (U.M.) and the fatty acids; on the other hand the values given for "fat" with the carcasses and gut represent the values obtained for the crude ether extract.

The cholesterol is estimated as the unsaponifiable fraction of the lipoids. The criticism might be made that this fraction is too ill-defined and includes compounds other than sterols. Preliminary experiments show, however, that the digitonin-precipitable material represents a high proportion of the U.M. (90% for the cholesterol-fed animals). It should be pointed out that the digitonin method shows a specificity for the normal series of sterols only; the *epi* series is not estimated by this method. Also as will be seen later coprosterol would be estimated by this method. The point at issue has been to decide whether cholesterol has been converted into a substance which would not be estimated in the unsaponifiable fraction and on this point the experiments yield suggestive results.

The question of the formation of coprosterol from cholesterol has not been studied but it will be realized that such a transformation would not affect the total sterols in the unsaponifiable matter isolated. Coprosterol can be readily isolated from the U.M. of the control faeces by recrystallization from acetone: water is added to the material dissolved in acetone until the solution becomes cloudy; it is then warmed and allowed to cool slowly; the first slight flocculent precipitate is filtered off and the solution left to stand at 4°; after some hours the coprosterol crystallizes out in needles; the crystals are filtered off rapidly on a chilled filter. After one recrystallization from acetone a material was obtained giving the following analytical figures (Weiler): C, 83·09; H, 12·40%. $C_{27}H_{48}O$ requires C, 83·33; H, 12·48%. The M.P. was 93·5°, and although this is lower than the value for synthetic material (102°) the M.P. was not depressed on admixture with an authentic sample of synthetic coprosterol obtained from Dr Rosenheim.

It has been found impossible to use the colorimetric method for cholesterol with any degree of accuracy. Samples of the various fractions have been kept and their analysis will be the subject of a further communication.

Analyses of the animals

The results obtained for the analyses of the livers, carcasses and guts of the animals on the various diets are shown in Table III. It will be observed that when fat is included in the cholesterol-containing diet "fatty" livers result. On a fat-free diet there is no increase of fat in the liver. It will also be seen that the results obtained with the triolein-containing diet are not consistent. In Exp. 6*a* the animal died and the amount of fat in the liver had not increased. This animal

Table III. *The analyses of the animals for "fat" or total lipoid and unsaponifiable matter* (U.M.) *at the conclusion of the experiments*

Exp. no.	Diet	Sex	Wt. of animal g.	Liver						Carcasses					Gut		
				Wt. g.	As % body wt.	"Fat" g.	U.M. g.	% "Fat"	% U.M.	Wt. g.	"Fat" (*a*) g.	U.M. (*b*) g.	*a* as % body wt.	*b* as % body wt.	Wt. g.	"Fat" g.	U.M. g.
1	Control arachis oil	♂	222	7·73	3·50	0·293	0·026	3·79	0·34	162·0	30·53	0·149	18·85	0·09	17·25	4·97	0·070
2 *a*	Plus cholesterol	♂	176	13·69	—	1·553	0·451	11·35	3·29	302·0	59·70	0·184	19·70	0·06	21·0	3·76	0·086
b			192														
3 *a*	Control arachis oil	♀	160	5·81	3·60	0·165	0·010	2·84	0·17	272·0	47·80	0·216	17·50	0·08	20·4	1·80	0·060
b			137	5·89	4·30	0·213	0·018	3·62	0·31								
4 *a*	Plus cholesterol	♀	112	5·40	4·80	1·253	0·440	11·30	3·97	188·0	36·60	0·250	19·45	0·13	16·4	1·70	0·140
b			117	5·70	4·90												
5 *a*	Control triolein	♂	144	7·40	5·10	0·297	0·027	4·02	0·36	251·0	32·20	0·710	12·80	0·28	19·7	0·62	0·126
b			145	6·90	4·80	0·292	0·032	4·23	0·46								
6 *a*	Plus cholesterol	♂	76	3·00	3·95	0·129	0·039	4·10	1·30	56·4	1·33	0·265	2·35	0·47	4·7	0·17	0·027
b			157	8·40	5·35	0·982	0·255	11·70	3·04	138·5	24·00	0·470	17·30	0·34	10·8	1·34	0·193
7 *a*	Plus cholesterol	♂	97	7·59	7·80	0·752	0·074	5·54	0·55	150·0	14·80	0·268	9·85	0·18	17·2	0·90	0·096
b			92	6·24	6·75												
8	Control beef dripping	♀	188 mean	6·39 mean	3·40 mean	1·094	0·097	5·63	0·50	—	—	—	—	—	—	—	—
9	Plus cholesterol	♀	231 mean	10·20 mean	4·40 mean	4·361	1·214	13·80	3·88	—	—	—	—	—	—	—	—
10	Control fat-free	♀	117 mean	4·63 mean	4·00 mean	0·536	0·054	3·86	0·39	303·0	17·60	0·940	5·80	0·31	23·1	2·92	0·127
11	Plus cholesterol	♀	133 mean	5·16 mean	3·90 mean	0·647	0·056	4·17	0·36	333·0	33·25	1·380	10·00	0·415	34·3	7·75	0·343

had not eaten for some days before its death. In Exp. 7 the amount of fat in the liver has increased slightly but the amount is not comparable with that observed in Exp. 6. Exp. 7 was actually carried out first but it was uncontrolled. The animals showed a severe diarrhoea which fact probably accounts for the relatively small absorption. In Exp. 6 and with the control animals there was an initial period of diarrhoea which lasted for about a week but thereafter the animals, with the exception of no. 6*a*, adapted themselves to the diet.

It will be seen from the table, and with the reservations concerning the triolein-fed animals, that cholesterol is absorbed on a fat-containing diet irrespective of the type of fat fed. Also from the results of Exps. 2 and 4 there is apparently no difference in behaviour of the two sexes.

Analysis of the faeces

The faeces were collected over the whole period and finally dried at 70° to approximately constant weight. They were then weighed and powdered. An aliquot was extracted in a Soxhlet apparatus with ether for at least 72 hr. It has been found that more complete if somewhat more highly coloured extracts are obtained by using this solvent in place of light petroleum. The results for the analysis of the faeces are given in Table IV. They are in agreement with the

Table IV. *Details of the analyses of the lipoid and unsaponifiable matter* (U.M.) *in the faeces of the rats fed on the various diets*

Exp. no.	Diet	Dry wt. faeces g.	Aliquot estimation: Amount g.	Aliquot estimation: Lipoid g.	Aliquot estimation: U.M. g.	Total in faeces: Lipoid g.	Total in faeces: U.M. g.	Lipoid as % faeces	U.M. as % lipoid
1	♂ controls arachis oil	59·7	20·0	2·62	0·93	7·8	2·97	13·0	38·1
			20·0	—	1·06				
2	Plus cholesterol	97·8	20·0	7·59	4·89	37·20	23·80	38·0	64·0
3	♀ controls arachis oil	79·4	20·0	2·16	0·84	8·60	3·23	10·8	37·6
4	Plus cholesterol	94·2	40·0	17·05	10·39	40·10	24·50	43·5	61·0
5	Controls triolein	21·9	20·0	2·71	1·26	2·96	1·38	13·6	46·5
6	Plus cholesterol	34·6	20·0	7·72	5·31	13·35	9·20	38·6	68·7
7	Plus cholesterol	29·3	29·3	9·92	6·63	9·92	6·63	33·9	66·7
8	Controls beef dripping	37·0	20·0	3·40	0·545	6·28	1·01	17·0	16·0
9	Plus cholesterol	52·1	20·0	7·96	3·82	20·74	9·96	39·8	48·0
10	Controls fat-free diet	22·2	10·0	0·79	0·45	1·75	1·00	7·9	57·0
11	Plus cholesterol	36·0	10·0	3·71	3·41	13·36	12·30	37·1	92·0

previously published results. The lipoid is the ether-soluble material. This was saponified for at least 5 hr. with 10% alcoholic KOH. The material was then diluted with water and extracted with ether. The ether extracts were washed 5 times with water. No soaps have been found in tested portions of the U.M. The remaining alkaline material was acidified with conc. HCl and the acids extracted with light petroleum. These results will be dealt with later.

Analysis of the urine

Qualitative experiments had indicated that the amount of cholesterol excreted in the urine of cholesterol-fed rats was not greatly augmented but no quantitative experiments had been made. The urines of the animals in Exps. 1, 2, 3 and 4 (Table II) were collected over the period of experiment and the results shown in Table V were obtained.

The urinary lipoids were estimated by first heating the urine on a water bath for 1 hr. with HCl and extracting with ether. The ethereal extracts were saponified and the U.M. extracted. The U.M. gave a precipitate with digitonin solution.

Table V. *Analyses of rats' urine*

Exp. no.		Vol. ml.	U.M. in aliquot	U.M. calculated in total vol. mg.
1	Normal	774	—	—
2	Cholesterol	596	—	—
3	Normal	1706	270 ml. gave 8·8 mg.	55·5
4	Cholesterol	630	200 ml. gave 17·4 mg.	54·8

A point of interest is the decrease in the urine volume of the cholesterol-fed animals. It was also noticed that the urine was much darker in colour. The results indicate that excretion of U.M. in the urine is a negligible factor in the metabolism of cholesterol although the reservation must be made that other breakdown products may be present.

The cholesterol (as U.M.) balance sheet

From the values that have been obtained it is now possible to construct a balance sheet (Table VI).

Table VI. *The U.M. balance sheet*

The values for U.M. in the table have been obtained by subtracting from the U.M. of the cholesterol-fed animals the amount of U.M. present in the control animals.

	Arachis oil		Triolein	Beef	
	♂	♀	Exp. 6	dripping	Fat-free
U.M. in	g.	g.	g.	g.	g.
Faeces	20·83	21·27	7·82	8·95	11·30
Livers	0·43	0·41	0·24	1·12	0·00
Carcasses	0·04	0·03	0·03	—	0·44
Gut	0·02	0·08	0·09	—	0·22
Total	21·32	21·79	8·18	10·07	11·96
Cholesterol fed	28·10	28·70	10·00	14·60	12·00
% accounted for	76·00	76·00	82·00	69·00	99·70

These results show that when fat is included in the diets of cholesterol-fed rats there is a loss of cholesterol irrespective of the type of fat fed or the amount of cholesterol. The loss of cholesterol varies between 20 and 30 %. The higher value was obtained in Exp. 8 and here the results are not complete as the U.M. was not determined in the carcasses or gut. It is extremely probable that here a higher percentage of cholesterol would have been recovered had these values been determined. The results for the fat-free diet are of interest. It will be seen that here there is virtually complete recovery of the cholesterol fed. The value for the carcass U.M. is rather high and might possibly be correlated with the fact that the cholesterol-fed animals grew better than the control animals. The results make it exceedingly probable that in the rat cholesterol is only metabolized in the presence of fat. It would also appear that the type of fat fed has but slight effect on the metabolism of cholesterol.

The fate of the cholesterol

Some indications of a possible fate of the cholesterol or some association with acidic substances may now be given. Table VII is what might be called the lipoid balance sheet of cholesterol metabolism as reflected in the faeces. It would appear from the results that have been given that the lipoid constituents of the carcasses do not give any clue as to the fate of the cholesterol.

In column A are the values for the extra faecal lipoids due to or caused by cholesterol feeding. In column B are the values for U.M. These figures appear in

Table VII. *The faecal lipoid balance sheet showing the difference in the distribution of the faecal lipoids of the control and cholesterol-fed animals on the various diets*

All values in g.

	Lipoid			U.M.			Diff. between A and B	Acids			Cholesterol			
Exp. nos. and diet	Controls	Plus cholesterol	Diff. A	Controls	Plus cholesterol	Diff. B	C	Controls	Plus cholesterol	Diff. D	Fed	Accounted for as U.M.	Metabolized E	% metabolized
1 and 2, Arachis oil ♂	7·80	37·20	29·40	2·97	23·80	20·83	8·60	4·45	10·10	5·65	28·10	21·32	6·78	24·1
3 and 4 Arachis oil ♀	8·60	40·10	31·50	3·23	24·50	21·27	10·20	5·23	10·38	5·15	28·70	21·79	6·91	24·1
5 and 6 Triolein	2·96	13·35	10·40	1·38	9·20	7·82	2·58	1·41	3·44	2·03	10·00	8·18	1·82	18·2
8 and 9 Beef dripping	6·28	20·74	14·46	1·01	9·96	8·95	5·51	4·80	8·25	3·45	14·60	10·10	4·50	30·4
10 and 11 fat-free	1·75	13·36	11·61	1·00	12·30	11·30	0·31	0·73	0·97	0·24	12·00	11·96	0·04	0·3

the U.M. balance sheet as faecal U.M. The difference between A and B shown in column C would therefore represent material other than U.M. caused to be excreted in the faeces by cholesterol feeding. This may be regarded as unaccounted lipoid. It will be obvious that this material should be present in the alkaline fraction as the potassium salt of an acid. If the alkaline fraction is acidified and extracted with light petroleum it is found that "fatty" acids are indeed present. In column D are given the differences between the amount of acids found in the faeces of the cholesterol-fed rats and those of the control animals. This can be regarded as the increase in the amount of acids caused by cholesterol feeding. In column E are given the values for the amount of cholesterol metabolized. This was obtained by subtracting the amounts of cholesterol recovered as shown in the balance sheet (Table VI) from the total amount of cholesterol fed in each separate series.

It will be seen from the table that the values shown in colums C, D and E in each experiment are of the same order. In Table VIII are given the results of previously published experiments [Cook, 1937] to which the amounts of acid have been added. These experiments are not complete because the acid was not estimated in the first series of faeces collected. A calculated value has therefore been added. The extraction of the faeces in these experiments was made with light petroleum.

Table VIII. *The lipoid balance in the faeces on varying fat concentrations plus cholesterol*

	Cholesterol			Lipoid unaccounted for	Acid found	% cholesterol metabolized
Diet fat %	Fed g.	Found in faeces and animals g.	Difference g.	g.	g.	
15	30·19	18·90	11·29	7·62	6·62	37·4
20	29·77	20·17	9·60	9·75	7·13	32·2
30	26·99	18·95	8·04	9·17	8·19	29·8
		Totals	28·93	26·54	21·94	
				Add calculated acid for first series	4·25	
					26·19	

It will be seen that here, as in the experiments described above, the missing cholesterol bears some relation to the production of acids which appear in the

faeces. It will be realized that if these acids are derived from cholesterol this might be reflected in an increase in the mean mol. wt. or equiv. wt. of the acids. The equiv. wt. has been determined by titrating with standard alkali in alcoholic solution at 60°. The results obtained are shown in Table IX.

Table IX. *The equiv. wt. of the mixed faecal fatty acids*

	Arachis oil	Triolein	Beef dripping	Fat-free
Controls	320 316	354 359 350	285 303	323 332
	333 320			
	Mean 322	Mean 354	Mean 294	Mean 327
Plus cholesterol	378 375	320 328 323	329 333	
			329 320	340
	Mean 375	Mean 323	Mean 328	

It must be emphasized that the values given are those of the mixed fatty acids and the results are comparative only. It will be seen that with the exception of the triolein and fat-free diets there is a tendency for the equiv. wt. of the acids to rise on cholesterol feeding. On the triolein and fat-free diets the equiv. wts. of faecal acids from the control animals are high in value. It is possible that with the arachis oil and beef dripping diets there is a selective excretion of higher fatty acids in association with the cholesterol. The appearance of these fatty acids was first observed on the arachis oil diet. The mixed acids were crystallized out of alcohol and on recrystallization an acid of M.P. 73·5° was obtained. On analysis this compound gave (Weiler): C, 78·86, 78·69 and 78·53, 78·58; H, 13·01, 12·97 and 13·03, 12·97 %. The mol. wt. (Rast) was 404 and the equiv. wt. 386 and 390. The compound did not take up bromine in alcoholic solution. The formula $C_{25}H_{50}O_2$ corresponds with the analytical figures but the substance is probably a mixture of high fatty acids. It is possible that this acid is derived from the "lignoceric acid"[1] which is present in relatively large amount in arachis oil. No systematic fractionation of the faecal acids has yet been made. A preliminary Twitchell separation indicates that the saturated acids form a high percentage of the material.

The results with the other fats cannot be so easily explained. The concentration of lignoceric acid in beef dripping is very low and triolein naturally does not contain any. The position is far from clear but it would appear that the excretion of cholesterol in the faeces, and possibly its metabolism, are associated with the appearance of acids of high mol. wt.

Summary

1. Rats have been fed on synthetic diets containing cholesterol in the presence of arachis oil, beef dripping and triolein. The cholesterol was also fed with a fat-free diet.

2. Cholesterol is absorbed irrespective of the type of fat fed. It is not absorbed on a fat-free diet.

3. Balance sheets of the cholesterol recovery on the various diets have been prepared. There is complete recovery on the fat-free diet but on the fat-containing diets only 70–80 % of the cholesterol can be recovered.

4. A lipoid balance sheet of the faecal excretion has been constructed. This indicates apparently that cholesterol metabolism is bound up with an increase of fatty acids in the faeces. The amount of cholesterol missing is approximately

[1] The position with regard to this acid in naturally occurring substances is well described by Chibnall *et al.* [1936].

equal in amount to the acids recovered. These acids are of high mol. wt. but it cannot be stated as yet that they are derived from cholesterol.

The writer is indebted to Sir F. G. Hopkins for his interest in the work. To Miss V. R. Leader he owes his thanks for tending the animals. To the Medical Research Council thanks are due for a grant covering part of the expenses.

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CLX. COENZYME FACTOR OF YEAST

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(*Received 26 May 1938*)

Animal tissues have been shown to contain coenzyme factor, an enzyme which catalyses the oxidation of coenzymes I and II by carriers [cf. Dewan & Green, 1937; 1938; Adler *et al.* 1937]. In this communication evidence is presented of the existence of the same enzyme in baker's yeast and other micro-organisms. The wide distribution of coenzyme factor offers additional evidence that it is the normal physiological agent for catalysing the oxidation of the pyridine nucleotides.

I. *Preparation of yeast coenzyme factor*

There are two methods available for extracting enzymes from yeast. The first and standard method is to dry yeast slowly, resuspend in water and allow autofermentation to proceed for some hours. By this procedure the more stable enzymes can be extracted. The method however is not applicable to the fragile enzymes like coenzyme factor and cytochrome oxidase which are destroyed in the course of drying the yeast. A more suitable method for obtaining these enzymes is that of the wet crushing mill (for a detailed description of the mill, cf. Booth & Green [1938]).

A suspension of baker's yeast (1 part yeast cake to 1 part water) was thoroughly ground in the roller mill and then centrifuged for 20 min. The supernatant liquid was mixed with $\frac{1}{2}$ vol. saturated $(NH_4)_2SO_4$ (*p*H 5) and was centrifuged. The supernatant fluid was discarded and the sediment was redissolved in the original volume of water. The precipitation with $(NH_4)_2SO_4$ was repeated twice. The precipitate was finally dissolved in the original volume of phosphate buffer, *p*H 7·2. By this procedure the coenzyme factor was obtained free from the soluble coenzyme dehydrogenases and from flavoprotein. Like its counterpart in animal tissues the yeast coenzyme factor is associated with highly peptized insoluble particles. As purification proceeds the insolubility of the particles becomes more noticeable. Sedimentation of the particles after the $(NH_4)_2SO_4$ procedure becomes feasible even in salt-free solution.

II. *The catalytic effect of yeast coenzyme factor on animal coenzyme dehydrogenase systems*

The coenzyme dehydrogenases catalyse the oxidation of their respective substrates by one of the two pyridine nucleotides. The reduced coenzyme is non-autoxidizable and its reaction with O_2 can only take place through the intermediation of some natural carrier such as cytochrome *a* or *b*, or an artificial carrier such as methylene blue. Coenzyme factor catalyses the reaction between reduced coenzyme I or II and the carrier. A system containing dehydrogenase, substrate, coenzyme, carrier and O_2 will not absorb O_2 unless coenzyme factor is added. Such a system is obviously suitable for testing the catalytic effect of coenzyme factor.

[1] Beit Memorial Research Fellow.

The preparation of the lactic, malic and triosephosphoric dehydrogenases of animal source free from coenzyme factor has already been described by Dewan & Green [1938]. Table I shows the effect of yeast coenzyme factor on these three systems. We have also been able to demonstrate the factor effect in the hexose-monophosphoric dehydrogenase system of rabbit skeletal muscle. Our colleague H. S. Corran will deal with the action of coenzyme factor on this system in a separate communication.

Table I. *The catalytic effect of yeast coenzyme factor on the lactic, triosephosphoric and malic dehydrogenase systems of animal tissues*

	μl. O_2 per 10 min.		
	Lactic	Malic	Triose-phosphoric
Complete system	189	181	165
Without factor	9	10	0
Without coenzyme	0	0	0
Without substrate	5	0	21
Without dehydrogenase	3	6	0

The following quantities were used in the complete systems; *lactic*—1·5 ml. dehydrogenase, 0·2 ml. factor, 0·7 ml. 0·2% coenzyme I, 0·2 ml. 2 *M* HCN, 0·05 ml. 0·5% methylene blue, 0·3 ml. *M*/3 pyrophosphate and 0·2 ml. *M* lactate; *malic*—same quantities except 0·2 ml. *M* malate instead of lactate; *triosephosphoric*—1·5 ml. dehydrogenase, 1 ml. factor, 0·7 ml. 0·2% coenzyme I, 0·2 ml. 0·5% methylene blue, 0·3 ml. *M*/3 pyrophosphate and 0·4 ml. *M*/10 hexosediphosphate (KOH in the centre pot).

III. *The catalytic effect of yeast coenzyme factor on yeast coenzyme dehydrogenase systems*

The malic, alcohol and triosephosphoric dehydrogenases were prepared from yeast by the following procedures.

Malic. The centrifuged juice of crushed baker's yeast was mixed at 0° with 3 vol. cold acetone. The precipitate was filtered and washed with acetone and ether. The dried powder was rubbed up with the original volume of water and the suspension was dialysed for 12 hr. at 0°. The precipitate was centrifuged and discarded. The solution was then treated for 10 min. at 52°.

Alcohol. Maceration juice of top brewer's yeast was diluted with 2 vol. water and dialysed for 6 hr. against running tap water. The centrifuged solution was then made 0·6 saturated with respect to $(NH_4)_2SO_4$, the precipitate collected by centrifuging and redissolved in the original volume of water. The $(NH_4)_2SO_4$ precipitation was repeated twice. The final solution was treated at 0° with an equal volume of cold acetone, and the precipitate was filtered and washed with acetone and ether. The dried powder was finally dissolved in the original volume of water.

Triosephosphoric. Dried baker's yeast was rubbed up with 10 vol. iced water and the suspension was centrifuged. This washing procedure was thrice repeated. The final suspension was made up by mixing 1 vol. of washed and centrifuged yeast cake with 2 vol. of water.

These three preparations contain highly active dehydrogenases free of coenzyme factor and flavoprotein. Table II shows the effect of addition of coenzyme factor to these dehydrogenase systems. It is clear that regardless of the source of the coenzyme dehydrogenase, whether from animal tissues or yeast, the reaction of reduced coenzyme I with methylene blue (or the natural carriers)

Table II. *The catalytic effect of yeast coenzyme factor on the malic, alcohol and triosephosphoric dehydrogenase systems of yeast*

	μl. O_2 per 15 min.		
	Malic	Alcohol	Triose-phosphoric
Complete system	212	134	235
Without factor	29	17	26
Without coenzyme	0	0	10
Without substrate	0	0	22
Without dehydrogenase	0	0	0

The following quantities were used in the complete systems: *malic*—1·5 ml. dehydrogenase, 0·2 ml. factor, 0·7 ml. 0·2% coenzyme I, 0·2 ml. 2 *M* HCN, 0·2 ml. 0·5% methylene blue, 0·3 ml. *M*/3 pyrophosphate and 0·2 ml. *M* malate; *alcohol*—1·5 ml. dehydrogenase, 0·5 ml. factor, 0·5 ml. 0·2% coenzyme I, 0·2 ml. 0·5% methylene blue, 0·3 ml. *M*/3 pyrophosphate, 0·2 ml. *M*/2 hydrazine and 0·1 ml. 98% alcohol; *triosephosphoric*—2 ml. yeast suspension, 0·5 ml. factor, 0·5 ml. 0·2% coenzyme I, 0·4 ml. *M*/10 hexosediphosphate and 0·3 ml. *M*/3 pyrophosphate (KOH in the centre pot).

takes place only in presence of the coenzyme factor. Furthermore, yeast factor works as efficiently with animal coenzyme dehydrogenase systems as does animal factor, and *vice versa*.

IV. *Comparison of yeast factor and flavoprotein*

Dewan & Green [1938] have shown that 1 mg. crude coenzyme factor of pig heart has the same activity as 20 mg. pure flavoprotein in the malic system (pig heart) and as 70 mg. pure flavoprotein in the triosephosphoric system (rabbit muscle). Tests with the crude yeast factor show that 1 mg. dry weight has the same activity as 6 mg. pure flavoprotein in the malic system (pig heart) and as 20 mg. pure flavoprotein in the triosephosphoric system (rabbit muscle). This discrepancy in no wise indicates that the coenzyme factors from the two sources are not the same. In the next section evidence is presented of the extraordinary similarity in properties of the enzymes from the two sources. It is important to note that the crude preparations of the factor whether from animal tissues or yeast contain all the insoluble enzymes and particles extracted from the cell. The coenzyme factor probably accounts for much less than 1% of the total dry weight. At this level of purity the absolute activity of the coenzyme factor is not very significant in a comparison of the enzymes prepared from different sources. What is significant however is that the catalytic activity of even the crude coenzyme factor is incomparably higher than that of pure flavoprotein.

The crude preparations of yeast factor were tested for the presence of flavoprotein with negative results. The fact that weight for weight the crude yeast coenzyme factor is many times more active than pure flavoprotein rules out all possibility that traces of flavoprotein could account for the catalytic effects. Flavoprotein was prepared from brewer's yeast by the method of Warburg & Christian [1932]. The flavin content was determined by extracting the dried powder with aqueous methyl alcohol and estimating the flavin content of the extract.

V. *Some properties of the yeast coenzyme factor*

Table III shows the extraordinary fragility of the yeast coenzyme factor. All these properties are identical with those of the coenzyme factor of pig heart. The yeast factor is also partially destroyed by drying and by precipitation with acetone.

Table III. *The effect of reagents and temperature on the activity of the yeast coenzyme factor*

Treatment	% loss in activity
10 min. at 52°	92
1 hr. at 38°	50
20 min. digestion with trypsin at 38°	100
3 min. at *p*H 4·0	75
3 min. at *p*H 4·6	0
3 min. at *p*H 9·0	90
12 hr. dialysis at 0°	10

Thus far we have been unable to detect any colour characteristic of the suspension of the coenzyme factor. The more concentrated suspensions are slightly brown but this colour does not change when the complete catalytic system is functioning. Dr Straub of the Molteno Institute kindly tested our preparations of the coenzyme factor for the presence of the coenzyme of the *d*-amino-acid oxidase. Only minute amounts were found to be present. As yet no direct evidence is available for assuming that the coenzyme factor contains the flavin-adenine coenzyme as its prosthetic group. Numerous attempts were made to resolve the coenzyme factor into a protein and crystalloidal component, but in no case was there evidence of such a resolution.

VI. *Coenzyme factor in other micro-organisms*

By a procedure similar to that employed in the case of baker's yeast and by the use of the roller mill it was possible to demonstrate the presence of coenzyme factor in the following micro-organisms: *Bact. coli*, *Bact. proteus* and *Bact. subtilis*. The most active preparation was obtained from *Bact. coli*.

Summary

1. The preparation of coenzyme factor from crushed suspensions of baker's yeast and other micro-organisms is described.
2. Like its counterpart in animal tissues the enzyme catalyses the oxidation by carriers of the reduced diphosphopyridine nucleotide (coenzyme I).

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CLXI. THE CONVERSION OF COLCHICINE INTO COLCHICEINE

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(*Received 26 May 1938*)

Colchicine is readily converted into the corresponding hydroxymethylene derivative, colchiceine, by hydrolysis of the methoxymethylene group in dilute acid solution. Colchiceine has only a fraction ($\frac{1}{40}$ to $\frac{1}{200}$) of the biological action of colchicine [Brues & Cohen, 1936; Ludford, 1936; Boyland & Boyland, 1937]. Hence the activity of orally administered colchicine might be partially destroyed by conversion into colchiceine by the action of the acid in the gastric juice.

Experimental

Method of estimation

Colchiceine gives a green colour with $FeCl_3$ in chloroform; colchicine and hexahydrocolchicine [Bursian, 1938] give no colour under the same conditions. The test can be used as a qualitative test for the detection of colchiceine in samples of colchicine. The method described below has been developed for the colorimetric estimation of colchiceine. As the colour reaction is given with trimethylcolchicinic acid, this and other hydroxymethylene ketones would be estimated by this method.

The solution containing colchicine and colchiceine is extracted by shaking with three separate lots of 3 ml. chloroform. The combined extracts are placed in a 15 ml. graduated flask, and 4 ml. absolute alcohol and 0·8 ml. of a 0·2 % solution of $FeCl_3$ in chloroform added. The total volume is brought to 15 ml. by addition of chloroform. Standards for comparison are made by dissolving known amounts of pure colchiceine in chloroform. Such standard solutions are unstable and must be freshly made. The colours developed by the unknown and an appropriate standard are compared in a colorimeter, using a red screen if the amount is less than 1 mg. The depths of colour obtained are easily compared without a screen if the amount of colchiceine present is 1–4 mg.

The greatest error in the method lies in the extraction of colchiceine from the aqueous solution with chloroform. Extractions of pure colchiceine showed that between 90 and 95 % with an average of 93 % was extracted. Allowance is made for this error in the calculation of results.

Hydrolysis of colchicine

Colchicine $C_{22}H_{25}O_6N$, 1·5 H_2O should theoretically yield 92 % of its weight of colchiceine $C_{21}H_{23}O_6N$, 0·5 H_2O. Colchicine, on heating in $N/10$ HCl at 100° for 1 hr., gives only 93 % of the theoretical amount of colchiceine (see Fig. 1), even after allowing for the loss in the extraction. By carrying out the hydrolysis for 1 hr. and then estimating colchiceine the original colchicine content of a solution can be determined. Hydrolysis in stronger acid such as N HCl gives lower yields

(see Fig. 1) owing to further decomposition and a similar decomposition occurs even in weaker acid.

Solutions containing 1 mg. colchicine per ml. were incubated with *N* HCl, *N*/50 HCl and with normal human gastric juice at 37°. Samples of the incubated solutions were taken and colchiceine estimated by the method described. The hydrolysis curves obtained are shown in Fig. 2.

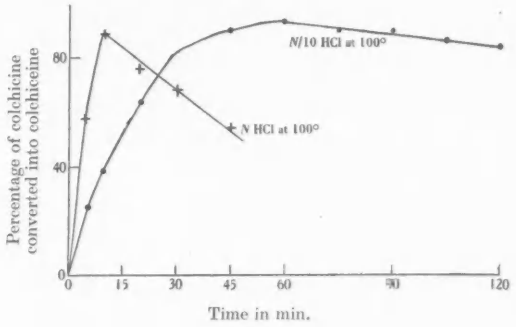

Fig. 1.

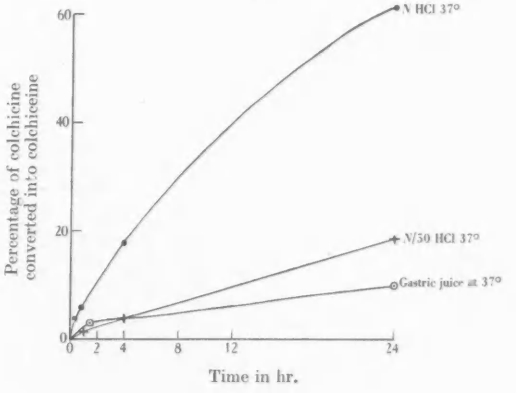

Fig. 2.

The amount of colchicine hydrolysed in *N*/50 HCl or by gastric juice during the time that orally administered colchicine would be expected to remain in the stomach is small. If colchicine is absorbed from the gut oral administration would be expected to be as effective as injection.

Summary

A method for the colorimetric estimation of colchiceine and colchicine is described. The rates of acid hydrolysis of colchicine at 37° and 100° have been measured.

We should like to thank the Sir Halley Stewart Trust for a studentship held by one of us (E. H. M.) and the British Empire Cancer Campaign for grants which have supported the work.

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CLXII. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

I. THE EFFECT OF CERTAIN ANTIBACTERIAL SUBSTANCES AND RELATED COMPOUNDS

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(*Received 1 June 1938*)

Many dyes containing aminonaphtholsulphonic acid residues have affinity for some cells contained in grafted tumours and lymph glands. Such dyes are isamine blue [Roosen, 1930], trypan blue [Opitz *et al.* 1926] and an azo dye formed from H-acid [cf. Kottman, 1935]. According to Ludford [1929] these dyes stain the macrophages of tumours rather than the malignant cells proper. Prontosil soluble, an antibacterial agent, contains an acetaminonaphtholsulphonic acid residue and on injection into animals with tumours it stains tumour tissue apparently in the same way as do the other dyes of this class. Prontosil soluble was found to have some slight inhibitory action on the growth of tumours in mice and many drugs related to this have been tested for their actions on the growth of tumours. The Schiff's base of 4:4′-diaminodiphenylsulphone and heptaldehyde was prepared and tested because heptaldehyde has been described as causing retrogression of spontaneous tumours [Strong, 1938].

The study of the effect of substances on tumour growth is much easier with grafted tumours as an unlimited number of comparable tumours can be obtained at any one time. With spontaneous tumours, however, each tumour must act as its own control and each has its own properties. It is also at present difficult to obtain sufficient spontaneous tumours.

Whereas grafted tumours become haemorrhagic on treatment with bacterial filtrates [Andervont, 1936] or colchicine [Boyland & Boyland, 1937] spontaneous tumours do not react in the same way. This difference in behaviour makes it appear possible that some substance might be effective against spontaneous cancers and not against grafted tumours. All substances mentioned in this paper have therefore been tested on both spontaneous tumours and grafted tumours but it is thought that more weight should be placed on the results with the spontaneous tumours.

Experimental

Benzidinesulphone was prepared as described by Culinane & Davies [1936]. Tolidinesulphone was prepared in the same way. Heptylideneaminobenzenesulphonamide was prepared by the general method of Gray *et al.* [1937]. It was crystallized from alcohol and had m.p. 171–172°. (Analysis (Schoeller) found: C, 57·3; H, 7·55; N, 10·15%. $C_{13}H_{20}SO_2N_2$ requires C, 58·0; H, 7·5; N, 10·20%.)

Female mice with spontaneous tumours which were either detected among the old breeding mice of the Strong A or Dilute Brown strains or supplied by dealers were examined and the tumours measured with calipers three times per week. The length and breadth of the tumour was recorded and the sum plotted. Normally the growth of tumours plotted in this way is linear [Boyland & McClean, 1935]. After the rate of growth of a tumour had been recorded for such a time

that the sum of length and breadth had increased by about 10 mm. the animal was treated as shown in the figures with the compound under investigation. All the substances were administered orally using a syringe with a long blunt needle. The toxicity of each substance was determined and the daily dose given was less than 1/4 of the toxic dose. Each substance was ground with water and gum tragacanth to give a suspension 0·5 ml. of which contained the daily dose. The results of the treatment with each compound were observed with four mice with spontaneous tumours as shown in the tumour growth curves. On the death of each mouse the tumour or tumours were removed for histological examination and in all cases were shown to be mammary carcinomata. The results are shown in Figs. 1–23 and summarized in Table I. The figures marked (*G*) are taken from Gray *et al.* [1937]. If the size of the tumour was less at the end of the period of treatment than at the beginning the tumour is described as having retrogressed.

Each substance was also tested in a similar way on the growth of the Crocker Sarcoma 180 in mice of the Strong A or Dilute Brown strains. In this case five mice of the same strain were treated with the compound while five mice grafted with the same tumour at the same time served as controls. If the substance produced definite inhibition of growth this is indicated by + in Table I.

Table I. *Action of substances on tumour growth in mice*

Substance	Toxic dose mg.	Dose given mg.	Effect on spontaneous tumours. Each substance tested on 4 mice: No. of tumours showing Inhibition of growth	Retrogression	Maximum survival under treatment days	Inhibitory action on grafted tumours (Crocker 180)
Prontosil soluble	>50	25	3	2	16	–
Prontosil rubrum	2 (*G*)	3	3	0	50	–
p-Aminobenzenesulphonamide	100 (*G*)	25	4	2	58	+
p-Heptylideneaminobenzenesulphonamide	200	25	3	2	28	–
p-Aminobenzenesulphonyl-*p'*-sulphonamidophenylamide hydrochloride	100 (*G*)	25	2	1	46	+
Sodium *o*-aminobenzenesulphonate	>200	50	2	2	21	+
Sodium *m*-aminobenzenesulphonate	>200	50	0	0	19	–
Sodium *p*-aminobenzenesulphonate	>200	50	4	1	43	–
Sodium sulphanilylsulphanilate	>200	20	4	3	33	+
Sodium *p*-hydrazinobenzenesulphonate	200	25	4	1	33	–
4:4′-Dinitrodiphenylsulphide	25	5	1	0	15	–
4:4′-Dinitrodiphenylsulphoxide	20	5	4	2	38	–
4:4′-Dinitrodiphenylsulphone	25	5	3	1	25	+
4:4′-Diaminodiphenylsulphide	30	5	3	1	35	–
4:4′-Diaminodiphenylsulphoxide	20	2	4	3	60	+
4:4′-Diaminodiphenylsulphone	10	2	4	2	16	+
4-*p*-Methoxybenzylidineamino-4′-aminodiphenylsulphone	200	4	3	3	15	+
4:4′-Diaminodiphenylsulphone glucose anil	100	10	4	2	37	+
Benzidinesulphone	20	4	3	0	44	+
Tolidinesulphone	10	4	3	2	15	–
Tetradecamethylene-1:14-diaminedihydrochloride	2	1	3	2	25	+
Synthalin A (decamethylene-1:10-diguanidine hydrochloride)	0·2	0·05	4	1	37	+
Synthalin B (dodecamethylene-1:12-diguanidine hydrochloride)	0·2	0·05	2	1	32	–

Some of the compounds were tested for any action which they might have upon the increase in body weight of young mice. Groups of four mice weighing 10 g. each were weighed daily and while one group remained as control other

groups were given *p*-aminobenzenesulphonamide, 4:4′-diaminodiphenylsulphone, 4:4′-diaminodiphenylsulphoxide and 4-*p*-methoxybenzylidineamino-4′-aminodiphenylsulphone. None of these compounds produced continued inhibition of body growth (see Fig. 24). Inhibition occurred with all of the compounds for periods of 3–9 days after the beginning of treatment but subsequently growth at the normal rate occurred, although the compounds were administered at the same level. The loss of weight which occurred during the first few days of administration of the drugs may be due to increase in the excretion of urine, such as occurs when *p*-aminobenzenesulphonamide is given to rats [Rimington, 1938].

Discussion

Although treatment with prontosil soluble (Fig. 1) produced inhibition of tumour growth, the mice did not survive the treatment very long; red prontosil (Fig. 2) and *p*-aminobenzenesulphonamide (Fig. 3) which are equally effective as antibacterial compounds appeared to be more effective in inhibiting tumour growth and animals so treated survived much longer. Heptylideneaminobenzenesulphonamide (Fig. 4) and *p*-aminobenzenesulphonyl-*p*-sulphonamidophenylamide hydrochloride (Fig. 5) also have some action of this kind.

Buttle *et al.* [1936] found that sulphanilic acid has only about 1/20 of the therapeutical action of *p*-aminobenzenesulphonamide and that *m*-aminobenzenesulphonic acid and *p*-hydrazinobenzenesulphonic acid had only slight actions against streptococcal infections in mice. Tested on tumours *m*-aminobenzenesulphonic acid had no action (Fig. 7) while *p*-aminobenzenesulphonic acid (Fig. 8) had definite action and *o*-aminobenzenesulphonic acid (Fig. 6) and *p*-hydrazinobenzenesulphonic acid (Fig. 10) some slight action. The marked difference between the *m*- and *p*-aminobenzenesulphonates indicates that some specificity in tumour-inhibiting action may be found. Sodium sulphanilylsulphanilate (Fig. 9) which has been recommended for treatment of canine distemper [Dochez & Slanetz, 1938] was more effective than sulphanilic acid or *p*-aminobenzenesulphonamide.

The 4:4′-diaminodiphenyl- and 4:4′-dinitrodiphenyl-sulphides, -sulphoxides and -sulphones (see Figs. 11–16) all showed some action but the most effective compounds were the sulphoxides, particularly 4:4′-diaminodiphenylsulphoxide. Levaditi *et al.* [1937] claim that 4-nitro-4′-aminodiphenylsulphoxide is particularly efficacious against gonococcal infections. In the case of one mouse (Fig. 14, no. 426) treatment with 4:4′-diaminodiphenylsulphide appeared to increase the rate of growth.

The Schiff's bases 4-*p*-methoxybenzylidineamino-4′-aminodiphenylsulphone (Fig. 17) and 4:4′-diaminodiphenylsulphone-glucose anil (Fig. 18) did not appear to be as effective as the original sulphones. Two diphenylene sulphones, benzidinesulphone (Fig. 19) and tolidinesulphone (Fig. 20), produced definite inhibition, although in these compounds the amino groups are situated in the *m*-position to the sulphone group. It is hoped to be able to test other substances related to benzidinesulphone.

Tetradecamethylene-1:14-diaminedihydrochloride, which King *et al.* [1937] have shown to be effective against some trypanosome infections, had some inhibitory action (Fig. 21) as also had synthalin A and synthalin B (Figs. 22 and 23) which Jancsó & Jancsó [1935] found to have trypanocidal action in mice.

In those cases in which the mice survived and measurements were made after treatment had been stopped, the rate of growth of the tumours returned to normal indicating that the compounds did not produce a permanent inhibition

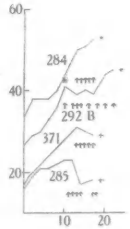


Fig. 1.

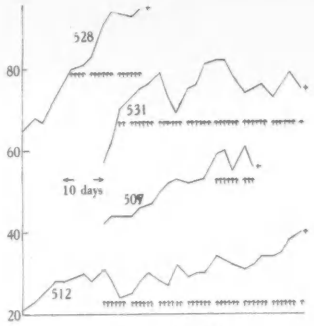


Fig. 2.

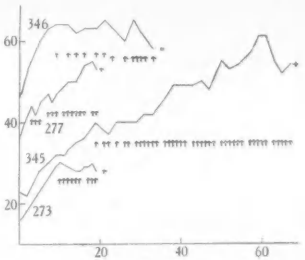
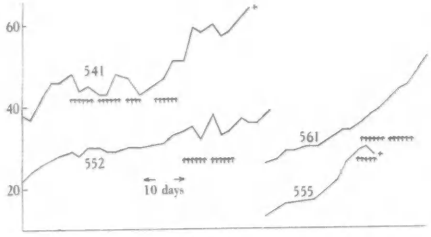


Fig. 3.



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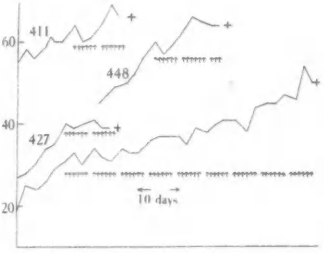

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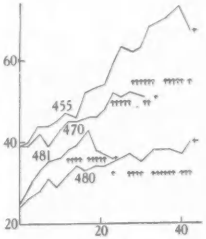

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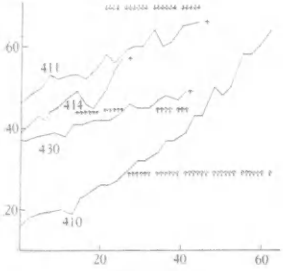

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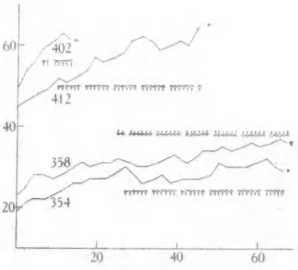
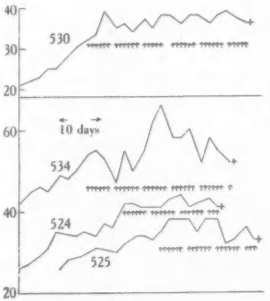


Fig. 8.



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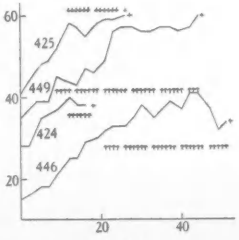

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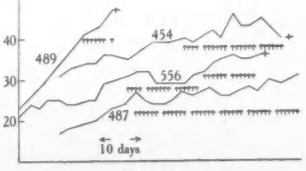

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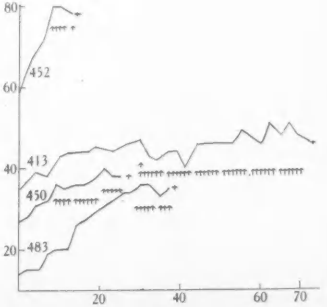
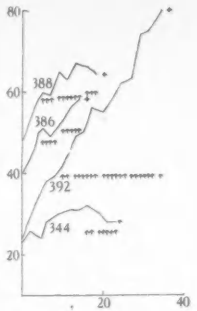


Fig. 12.

For explanation of Figs. 1–12 see p. 1213.



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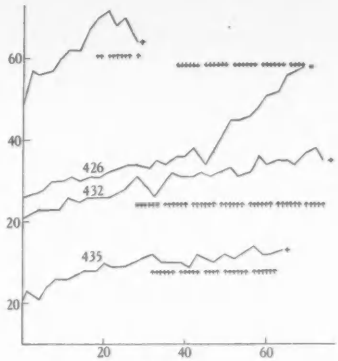

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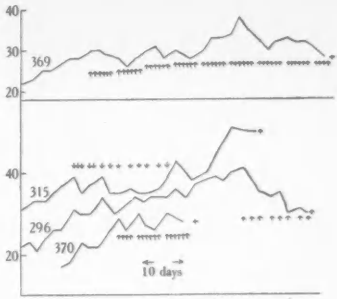

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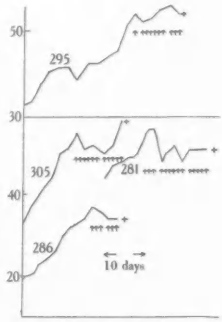

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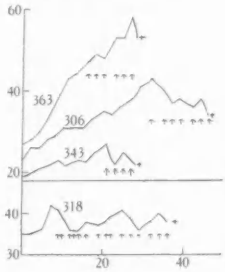


Fig. 17.

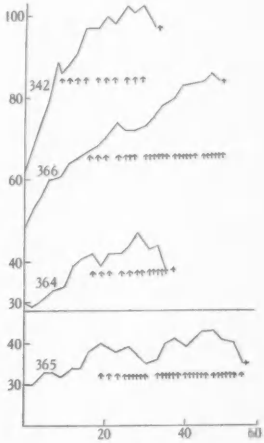
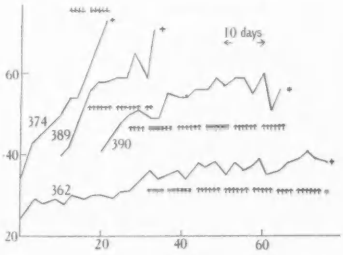


Fig. 18.



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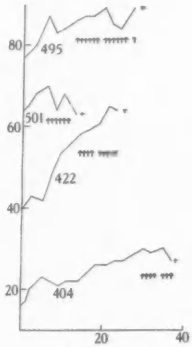

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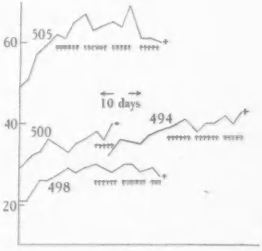
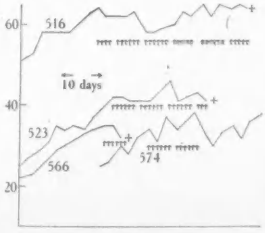


Fig. 21



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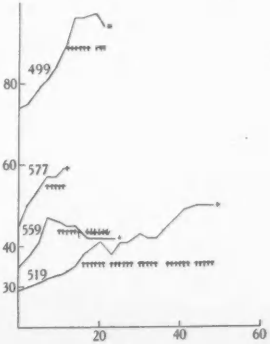

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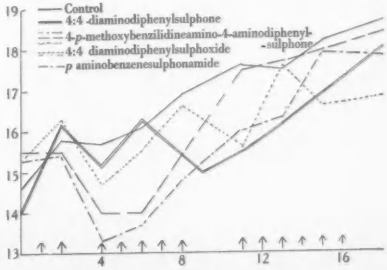

Fig. 24.

For explanation of Figs. 13–24 see p. 1213.

of growth. This effect is to be seen in Fig. 4 (mouse 541) treated with *p*-heptylideneaminobenzenesulphonamide, Fig. 15 (mouse 315) with *pp'*-diaminodiphenylsulphoxide and Fig. 22 (mouse 574) treated with synthalin A.

The effect of these substances on growth in body weight (Fig. 24) and the fact that the growth of tumours is inhibited only while the drugs are administered indicate that the inhibition is somewhat different from that produced by carcinogenic compounds [Haddow *et al.* 1937]. Carcinogenic hydrocarbons generally exert a prolonged inhibitory action both on tumour growth and on growth of body weight.

Many of the treated tumours in which growth was inhibited became much softer. The degree of hardness of a tumour is difficult to assess but the softened tumours vary considerably in size from day to day. As a result of this, marked variations in tumour size from day to day are recorded as shown in Fig. 2, mice 507 and 531; Fig. 4, mouse 552; Fig. 5, mouse 434; Fig. 9, mice 525 and 534; Fig. 10, mouse 446; Fig. 12, mouse 413; Fig. 15, mouse 370; Fig. 21, mouse 281; Fig. 17, mouse 318; Fig. 23, mouse 342 and Fig. 19, mice 362 and 390.

From Table I it can be seen that of the compounds tested only 4:4'-diaminodiphenylsulphoxide and sodium sulphanilylsulphanilate inhibited the growth of the Crocker sarcoma, inhibited the growth of all spontaneous tumours used and caused retrogression in the case of three out of four of the mice with spontaneous tumours. Other compounds related to these are being investigated.

Summary

Some aromatic sulphur compounds, have been found to retard the growth of spontaneous mammary cancer in mice. The most effective of the compounds tested appeared to be *pp'*-diaminodiphenylsulphoxide and sodium sulphanilylsulphanilate.

I should like to thank Mrs Bonser of the Department of Experimental Pathology and Cancer Research of the University of Leeds for supplying many of the mice with spontaneous tumours, Dr Smith of the Wellcome Chemical Works, Dr Henry and Mr Gray of the Wellcome Chemical Research Laboratories, and Dr H. King of the National Institute for Medical Research for their kindness in supplying many of the substances which have been tested. I am indebted to Messrs Schering for samples of synthalin A and synthalin B and Messrs Bayer Products for prontosil. The work has been supported by a grant from the British Empire Cancer Campaign to this Hospital.

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EXPLANATION OF FIGS. 1–24.

Abscissae: Figs. 1–24: Days.

Ordinates: Figs. 1–23: Tumour size as sum of length and breadth in mm.
Fig. 24: Average wt. of mice in g.

Fig. 1. Growth of spontaneous mammary tumours in mice treated with 7-acetamino-2:4′-sulphonamidobenzene-azo-1-naphthol-3:6-disulphonate (prontosil soluble).
In this and other figures each arrow represents the administration of the dose shown in Table I.

Fig. 2. Growth of mammary tumours in mice treated with 4-sulphonamidobenzene-azo-2′:4′-diaminobenzene (red prontosil).

Fig. 3. Growth of mammary tumours in mice treated with *p*-aminobenzenesulphonamide.

Fig. 4. Growth of mammary tumours in mice treated with *p*-heptylideneaminobenzenesulphonamide.

Fig. 5. Growth of mammary tumours in mice treated with *p*-aminobenzenesulphonyl-*p*′-sulphonamidophenylamide hydrochloride.

Fig. 6. Growth of mammary tumours in mice treated with sodium *o*-aminobenzenesulphonate.

Fig. 7. Growth of mammary tumours in mice treated with sodium *m*-aminobenzenesulphonate.

Fig. 8. Growth of mammary tumours in mice treated with sodium *p*-aminobenzenesulphonate.

Fig. 9. Growth of mammary tumours in mice treated with sodium sulphanilylsulphanilate.

Fig. 10. Growth of mammary tumours in mice treated with sodium *p*-hydrazinobenzenesulphonate.

Fig. 11. Growth of mammary tumours in mice treated with 4:4′-dinitrodiphenylsulphide.

Fig. 12. Growth of mammary tumours in mice treated with 4:4′-dinitrodiphenylsulphoxide.

Fig. 13. Growth of mammary tumours in mice treated with 4:4′-dinitrodiphenylsulphone.

Fig. 14. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphide.

Fig. 15. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphoxide.

Fig. 16. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphone.

Fig. 17. Growth of mammary tumours in mice treated with 4-*p*-methoxybenzylideneamino-4′-aminodiphenylsulphone.

Fig. 18. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphone glucose anil.

Fig. 19. Growth of mammary tumours in mice treated with benzidinesulphone.

Fig. 20. Growth of mammary tumours in mice treated with tolidinesulphone.

Fig. 21. Growth of mammary tumours in mice treated with tetradecamethylene-1:14-diaminedihydrochloride.

Fig. 22. Growth of mammary tumours in mice treated with decamethylenediguanidine hydrochloride (synthalin A).

Fig. 23. Growth of mammary tumours in mice treated with dodecamethylenediguanidine hydrochloride (synthalin B).

Fig. 24. The growth in body weight of mice treated with certain sulphur compounds.

CLXIII. FAT METABOLISM IN FISHES

XIII. FACTORS INFLUENCING THE COMPOSITION OF THE DEPOT FAT OF FISHES

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(*Received 23 May 1938*)

THERE are at least four factors which possibly affect the composition of the depot fat of a fish. These are (1) species, (2) diet, (3) and (4) temperature and salinity of the water.

Species. It is well known that different species of animals lay down depot fats more or less characteristic of that animal. With normal diet the composition of the depot fat will remain roughly the same. In the case of fish the author [Lovern, 1937] has shown that various characteristic depot fats are produced from a roughly uniform diet, according to species, their composition thus being largely independent of diet.

Diet. It is equally well known that the diet of an animal may affect the composition of its depot fat. The process, however, is by no means quantitative, and when it is studied in detail some contradictory results are obtained. There are two types of experiment which throw light on this problem.

In the first type certain fatty acids are fed which can be detected if they are incorporated in the depot fat, e.g. elaidic acid and acids containing deuterium. Schoenheimer & Rittenberg [1935] found that in mice fed for 4 days on a diet containing 1 % of fat containing deuterium (the only fat in the diet), 47 % of the ingested fat was found in the depots. The authors conclude that the largest part of the dietary fat, even at this low level of ingestion, is deposited in the fatty tissues before it is used. In another experiment the same workers [Rittenberg & Schoenheimer, 1937] find evidence of a continual turnover between depot fat and ingested fat and suggest a half-lifetime for depot fatty acids of 5–9 days.

In the second type of experiment fats are fed which differ from the normal depot fat of the animal in the proportions of the different acids present, or in the unsaturation of some of these. Here the results obtained are quite different and sometimes contradictory. Thus, Thomas *et al.* [1934] found that liberal allowances of whole soybeans, menhaden oil, corn oil and coconut oil fed to steer calves for 260 days had no perceptible effect on the unsaturation of the body fats. On the other hand, Ellis [1933] found a marked decrease in saturation in pig's fat after feeding peanuts and an increase in saturation after feeding corn. Possibly the different species of animal used has something to do with this apparent contradiction. It should also be remembered that whilst a change in iodine value indicates a change in composition, a constant iodine value does not necessarily connote a constant composition.

The experiments of this second type uniformly indicate that fat must be fed at a fairly high level before any considerable effect is produced on the body fat. Thus, Banks & Hilditch [1932] found 1–2 % of C_{20} and C_{22} unsaturated acids in the fat of a pig fed on a diet containing 0·7 % of fish fat. Brown [1931] fed pigs on a diet containing 14 % of menhaden oil, but the content of C_{20} and C_{22}

unsaturated acids in the fat was only 2·7 %, so that all this extra fish fat had had little effect on the depot fat. Bhattacharya & Hilditch [1931] showed that 3 % of shea butter or of arachis oil in the diet of a pig made little difference to the proportions of the various acid groups, and suggested that acids ingested in excess are disposed of (by oxidation) to leave a residue "more or less adapted to the requirements of the particular storage fat". Again, Banks *et al.* [1933] found that when about 5 % of cod liver oil was included in the diet of rats, there was not much alteration in the depot fat, apart from some deposition (about 8 %) of C_{20} and C_{22} acids. The rest of the acids appeared to be present in much the same relative proportions as when the diet was mainly fat-free. Therefore the ingested cod liver oil had not been freely incorporated in the rat's depot fat.

The discrepancy between the results obtained by experiments of these two types may be understandable. There are two characteristics of a fatty acid which may affect a fat in which it is incorporated—unsaturation and length of carbon chain. It may be that the requirements of an animal with regard to the chain lengths of its depot fatty acids are specific. In the case of fish, where there is a great range of chain lengths amongst the various fatty acids, the relative proportions of these groups of acids are often quite characteristic of the species. Now a fatty acid containing deuterium, for instance, might well become incorporated in the depots in place of some normal acid of the same chain length without upsetting the animal's specific requirements. Whatever the reason, there seems little doubt that whilst normally there is constant interchange between ingested fat and depot fat (as shown by the deuterium experiments), this is not unconditionally the case when a fat with unusual proportions of the various acids is fed in the diet. Some selective mechanism then operates, and surplus ingested acids of various chain lengths are eliminated or modified until a mixture resembling the normal depot fat remains. If, however, the unusual fat fed is above a certain limit this selective mechanism partly breaks down.

There has been very little work done on the effect of different dietary fats on fish.

Temperature. It is generally accepted that temperature has an effect on the composition of the fats of both animals and plants, higher temperatures tending to produce more saturated fats. It should be noted that this only applies to a given species under varying conditions of temperature. This effect has been studied, for example, in pigs, and Dean & Hilditch [1933] have confirmed and amplified the earlier results of Henriques & Hansen [1901]. In the case of fish, Brocklesby & Bailey [1932] found an increasing iodine value in the body oil of the salmon as they went further north (provided that they kept to the same species of salmon), and stated that this corroborated the findings of investigators working on other species of fish. Tropical fish do not necessarily have a more saturated fat than other (even closely related) species inhabiting colder waters [Lovern, 1932; 1935, 2].

Salinity. It has been shown repeatedly [Lovern, 1937] that fresh-water fish have fat different from that of marine fish, but the reason has not yet been found. The difference is also found in the vegetable-feeding zooplankton [Lovern, 1935, 1]. These small Crustacea must produce these characteristic types of fat by appropriate modification of ingested fat, at least in the case of the marine organisms [Lovern, 1936]. Whilst this effect is apparently associated with salinity alone, it may be an indirect effect. Thus, Fox & Simmonds [1932] have found that the oxygen uptake of Crustacea is greater in fresh water than in salt water, and the whole metabolic rate is higher.

In the experiments described in this paper an attempt has been made to study the individual effects of diet, temperature and salinity on the fat of a suitable species of fish. The common eel, *Anguilla vulgaris*, was chosen because (*a*) it has a high fat content and adequate quantities of material can be obtained from a reasonable number of individuals, and (*b*) it can live in fresh water or sea water.

Experimental

The apparatus consisted of three large tanks, each of which was connected to a smaller tank. The small tanks, placed below the larger ones, were fitted up as thermostats with both heating and cooling appliances. Water ran continually through gravel filters from the upper into the lower tanks, and was pumped back as an aerated froth. The eels were confined in the large tanks. They were fed daily, and each day the water was changed, affording an opportunity for cleaning the tanks and removing unconsumed food. Refilling was done slowly through the thermostatic tanks to minimize temperature disturbances.

In the first experiment the tanks were set up as follows: 1, warm sea water; 2, warm fresh water; 3, cold fresh water. Each tank was stocked with 50 eels 30–35 cm. in length. Until feeding had commenced the temperatures were maintained at about 15°. In fresh water the eels refused food for about 10 days, but in sea water they commenced feeding at once. The diet consisted of shelled mussels (*Mytilus edulis*) and was fed *ad lib.* After feeding had become general, the temperatures were adjusted to the highest and lowest limits at which feeding was satisfactory. On raising the temperature progressively from 15°, the daily consumption of food increased until a temperature of 23° was attained. After this point consumption declined, and so this temperature was maintained for the two warm-water tanks. Similarly, on lowering the temperature from 15° feeding was diminished, and below 12° almost ceased. A temperature of 14° was selected as the most suitable for the cold-water tank and was maintained throughout the experiment.

Unfortunately, in this first experiment, no record was kept of increases in weight or of food consumption. Each month a sample of mussels was examined for fat content; there was little variation, the total ether-soluble matter averaging about 1·35 %. Feeding was continued for 6½ months, when the eels were killed and the body fats extracted. In all the tanks there were a few deaths due to initial injury and to fighting, and in two cases (after 3 months in warm sea water and 5 months in warm fresh water) there was a breakdown of aeration which caused the deaths of about 20 eels in each case, the remainder in each tank recovering. These latter dead eels were also extracted and their body fats examined.

As stated, the eels were originally all about 30–35 cm. in length. It is necessary to have a uniform length to get roughly uniform fat content. The fat content of the eel increases almost linearly with length, at any rate between certain limits (Fig. 1). Thus, on the average, these eels would start with about 9 % of fat.

At the end of the experiment some individuals appeared to have grown appreciably, whilst others had not. It is known that the so-called "male" eels do not grow so fast as the female eels, and possibly the differences observed were due to mixed "male" and female eels being used. There was a greater preponderance of larger eels in tank 3 at the end of the experiment than in the other tanks.

The fat contents of the eels are given in Table I and the composition of the fats in Table II. A control sample of eels of the same length obtained from the same source (estuarine waters of the River Dee), is included in both tables.

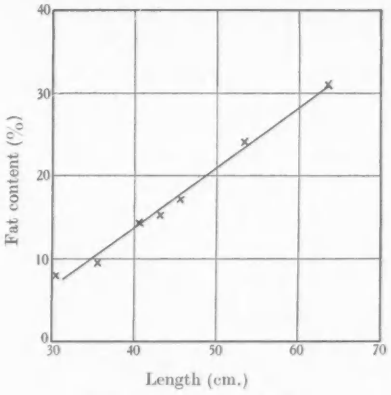

Fig. 1. Fat content of eels.

Table I. *Fat contents of eels*

Batch no.	Description	Period fed months	Fat content %	Iodine value
1	Control	—	9·3	118·5
2	Fresh water 14°	$6\frac{1}{2}$	18·0	119·1
3	,, 23°	5	8·2	126·4
4	,, 23°	$6\frac{1}{2}$	10·2	121·5
5	Sea water 23°	3	8·9	118·5
6	,, 23°	$6\frac{1}{2}$	9·4	116·6

Table II. *Composition of fatty acids of eel fats (wt. %)*

Batch no.	Saturated			Unsaturated				
	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
1	4·3	16·8	2·5	0·1	8·8 (−2·2 H)	39·4 (−2·5 H)	20·8 (−5·6 H)	7·3 (−10·2 H)
2	3·3	20·5	2·7	Nil	11·6 (−2·4 H)	39·4 (−2·7 H)	16·1 (−6·3 H)	6·4 (−10·0 H)
3	3·6	17·9	2·8	Trace	6·8 (−2·3 H)	39·2 (−2·6 H)	22·4 (−5·8 H)	7·3 (−8·0 H)
4	4·1	19·4	2·2	Nil	5·7 (−2·1 H)	37·5 (−2·8 H)	20·6 (−5·5 H)	10·5 (−9·2 H)
5	3·1	19·8	4·8	0·7	5·8 (−2·4 H)	42·6 (−2·7 H)	15·7 (−5·5 H)	7·5 (−8·0 H)
6	3·7	17·4	3·9	Nil	9·5 (−2·1 H)	39·6 (−2·4 H)	16·8 (−5·2 H)	9·1 (−8·6 H)

A large quantity of mussels was extracted, and the ether-extract (123 g.) separated into acetone-soluble fat (73 g.) and acetone-insoluble crude phosphatides (50 g.). The mixture of fatty acids recovered from each fraction was analysed. These mussels were obtained in December 1936. In October 1937 (in connexion with a second experiment) another, larger batch of mussels was extracted, giving 324 g. acetone-soluble fat and 152 g. phosphatides. The original analysis on the fat fraction was satisfactory, but the analysis of phosphatides

was deemed unreliable, so another analysis was carried out on the second batch of phosphatides. In each case appreciable amounts of organic acids of non-fatty nature were encountered, also about 20 % of highly unsaturated acids of high molecular weight (about C_{28} ?). Both phosphatide acid analyses are only approximate in view of the considerable experimental difficulties. These unusual acids did not occur in the acetone-soluble fat. The figures for the mussel lipoids are given in Table III (with non-fatty acids and C_{28} (?) acids excluded).

Table III. *Fatty acids of mussel lipoids (wt. %)*

	Saturated			Unsaturated					
Material	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
Fat	1·9	16·7	1·7	0·3	10·6 (−2·5 H)	21·5 (−4·1 H)	29·9 (−7·3 H)	13·9 (−9·3 H)	3·5 (− ? H)
Phosphatide (1)*	Nil	28·5	14·4	Nil	3·2 (−3·5 H)	10·1 (−4·6 H)	29·9 (−6·4 H)	13·2 (−8·6 H)	? —
Phosphatide (2)	Nil	27·3	6·2	Nil	5·0 (−3·2 H)	16·4 (−4·0 H)	32·2 (−6·0 H)	12·9 (−8·5 H)	? —

* Plus 0·7 % arachidic acid.

This first experiment was considered unsatisfactory in several respects—in particular, the lack of records of growth and food consumption, and the use of mixed "male" and female eels with different growth rates. A second experiment was undertaken with the conditions as follows.

(1) The tanks were filled with (*a*) sea water at 14°, (*b*) fresh water at 14°, (*c*) fresh water at 23°.

(2) Each tank was stocked with 25 eels 50–55 cm. in length. This is above the supposed maximum size for "male" eels.

(3) The combined weight of eels in each tank was taken at the start and finish of the experiment.

(4) The diet in the fresh-water tanks consisted of herring. A large supply of herring in uniform condition was obtained at the start of the experiment. This was kept in cold storage and the required amount withdrawn daily. It was fed as pieces of flesh (with skin) about 2 cm. square, containing 20·7 % fat. The diet in the sea-water tank consisted of mussels.

(5) The food was fed *ad lib.*, and the daily consumption was measured.

(6) Any eels which died during the course of the experiment (which ran for 6 months) were weighed and the weight was subtracted from the initial weight. It was assumed that they had eaten nothing since the commencement of the experiment, which was in most cases probably true, owing to initial injuries.

The particulars of the eels are given in Table IV and the composition of their fat in Table V. A control sample of eels of 50–55 cm. length, obtained from the estuary of the Dee, was also examined. The composition of the fat of the herring used is included in Table V. The samples are numbered as follows: 1, control; 2, sea water at 14°; 3, fresh water at 14°; 4, fresh water at 23°. The initial weights are the corrected ones, allowing for 10 deaths in sample 2, 7 deaths in sample 3, and 8 deaths in sample 4.

Table IV. *Particulars of eels in Exp. 2*

Sample	Initial wt. g.	Final wt. g.	Total food eaten g.	Final fat content %	Iodine value
1	—	—	—	24·1	119·0
2	3038	2574	10,459	16·6	114·1
3	2732	3159	4,140	20·6	140·4
4	2829	3556	5,739	23·9	137·6

Table V. *Fatty acid compositions of eel fats in Exp. 2 (wt. %)*

	Saturated			Unsaturated				
Sample	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
1	4·3	17·8	1·7	Trace	9·2 (−2·2 H)	38·4 (−2·7 H)	20·1 (−6·0 H)	8·5 (−9·3 H)
2	4·8	17·6	2·6	—	9·4 (−2·2 H)	41·6 (−2·4 H)	16·4 (−5·8 H)	7·6 (−9·1 H)
3	6·4	17·1	1·3	—	6·9 (−2·4 H)	31·9 (−3·0 H)	22·2 (−5·4 H)	14·2 (−7·5 H)
4	6·0	16·5	1·1	0·6	8·3 (−2·2 H)	33·9 (−2·8 H)	22·6 (−5·5 H)	11·0 (−7·0 H)
Herring	8·3	12·1	0·3	0·5	6·4 (−3·4 H)	21·0 (−4·5 H)	28·3 (−5·5 H)	23·1 (−4·6 H)

The eels in the cold sea water were fed on mussels to obtain data on increase (or loss) in weight on a diet so low in fat. In Exp. 1 there was reason to suspect considerable deposition of fat in the cold fresh water, judging by the unusually high fat content (18 %) of the eels. This was the more remarkable since the eels in cold water eat less of any given food than those in warm water. Actually, there was a loss in weight (as well as a reduction in fat content) on a similar diet in cold sea water (Table IV).

An attempt was made to keep eels in sea water on a diet of herring in a smaller tank than the three described. The water was not circulated, but was well aerated and changed daily. For some reason the eels refused to eat anything but particularly tempting food, such as live earthworms. After 5 months' total starvation the attempt was abandoned.

Discussion

It will, perhaps, be best if the influence of the various factors, diet, temperature and salinity, are discussed under separate headings, although some overlapping is inevitable.

Diet. The diets fed, mussels and herring, probably represent about the extremes of non-fatty and fatty food likely to be eaten by an eel in its natural environment. The mussels contained on an average 1·35 % of ether-soluble matter. Allowing for the 15 % of unsaponifiable matter present in this and for the lower proportion of fatty acids in phosphatides than in true fats, the mussels contain about 1·1 % of fat. Not many aquatic organisms will have appreciably less fat than this, and not many will have more than the 20·7 % of fat present in the herring.

Both the dietary fats differ considerably in composition from eel's fat. In the mussel, considerable proportions of phosphatide are present. The acids of the neutral fat and of the phosphatides differ mainly in the presence of more palmitic and stearic acids and less C_{16} and C_{18} unsaturated acids in the phosphatides. If total groups are considered (all C_{16} acids, for instance, being considered together), there is no marked difference between the fatty acid compositions of the three materials included in Table III. Indeed, one could imagine that the glycerides and phosphatides of the mussel had been formed from almost the same mixture of acids, with subsequent selective hydrogenation of the phosphatide mixture. The main differences between the mussel's fatty acids and those of the eel (no. 1 of Table II and also of Table V are very similar) are in respect of the presence of considerably less C_{18} acids, less C_{14} and more C_{20} and

C_{22} acids in the mussel. The most marked differences between herring's fat and eel's fat are in respect of the presence of much more C_{22} acids and much less C_{18} acids in the former. All the other groups, however, are appreciably different in the two fats, and there are more C_{14} and C_{20} and less C_{16} (especially total C_{16}) acids in the herring's fat.

The depot fat of the eels should have been appreciably modified if there had been any considerable incorporation of the ingested fat. From Table II, and also from comparison of samples 1 and 2 in Table V, it can be seen that there was no tendency for the fat of eels on a diet of mussels to be modified in the direction of mussel's fat. This is in accordance with evidence already quoted, that dietary fats differing from the fat of the experimental animal in the relative proportions of the various acids must be fed above a certain level before modification takes place.

On the diet of herring there has been appreciable modification of the eel's fat in the direction of herring's fat (Table V, samples 1, 3 and 4). In fact, the final compositions attained in samples 3 and 4 correspond well with those of definite mixtures of eel's fat and herring's fat. In Table VI are given the calculated compositions of mixtures of 62 % eel's fat with 38 % herring's fat and 78 % eel's fat with 22 % herring's fat, expressed as total groups, compared with the actual fats of samples 3 and 4 (Table V) respectively. The analytical method used is not sufficiently accurate, of course, to differentiate between, say, a 60/40 mixture and a 62/38 mixture. Furthermore, the original eel's fat and the ingested herring's fat were probably not absolutely uniform in composition.

Table VI. *Mixtures of eel and herring fats*

Material	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
62/38 mixture	6·0 (+)	23·8	33·0	23·2	14·0
Sample 3	6·4	24·0	33·2	22·2	14·2
78/22 mixture	5·3	25·1	36·0	21·9	11·7
Sample 4	6·6	24·8	35·0	22·6	11·0

It is evident from Table VI that when the ingested fat does find its way into the fat depots of the eel, it does so with the relative proportions of its various acids practically unaltered. Schoenheimer & Rittenberg's [1935] experiments suggest a deposition of most of the ingested fat in the depots before use, and other experiments [Rittenberg & Schoenheimer, 1937] suggest a continual turnover between depot fat and ingested fat. Whilst this may be true for normal dietary fats, it cannot be unconditionally so for dietary fats differing appreciably from the depot fat of the animal. The results with the eel demonstrate this. On the theory of continual turnover it is possible to calculate the final composition of the eel's fat by assuming that all the ingested fat is deposited in the depots and then a certain amount of the mixed fat is withdrawn.

Errors in the calculation may be caused by the assumption that all the eels in Exp. 2 started with about 24 % of fat. Fig. 1 was compiled from results obtained on batches of about 20 eels of each size. As the experimental batches consisted of a fair number of eels in each case, there is a good chance of individual variations being largely compensated. Moreover, it is unlikely that the average of all three batches varied much from 24 %. For the sake of the following calculations a uniform initial fat content of 24 % in Exp. 2 will be assumed.

From Table IV we can calculate the daily intake of fat and the daily retention. It will not cause serious error to assume a constant daily intake of 1/180th of the total amount eaten. On this basis sample 2 consumed 0·64 g. of mussel's

fat daily, and lost 301·4 g. of their own initial fat, or a total daily loss of 2·31 g. If this 0·64 g. was deposited unchanged in the depots and 2·31 g. of the mixed fat was withdrawn, the final composition after 180 days should be 81% eel's fat, and 19% mussel's fat. Such a mixture does not differ from eel's fat sufficiently to be detectable by analysis. Actually, the fat of sample 2 differs slightly from that of sample 1 in the reverse direction from that produced by admixture of mussel's fat with eel's fat. This is presumably due to variability in eel's fat itself and to experimental error. The apparent failure of a diet low in fat to affect the composition of the depot fat, may, therefore, in this instance at least, be due to the limitations of the analytical procedure.

Sample 3 ingested 4·76 g. of herring's fat daily and the daily loss of fat was 4·78 g. By the above hypothesis this process would finally give a mixture of 28% eel's fat and 72% herring's fat, contrasted with the 62% eel's fat: 38% herring's fat mixture actually found. Again, in sample 4 there was a daily ingestion of 6·60 g. of herring's fat and a daily loss of 5·65 g., which should ultimately give 24% eel's fat and 76% herring's fat, contrasted with the 78% eel's fat: 22% herring's fat mixture found. Such differences could readily be detected by analysis.

The differences are so large that it seems improbable that they can be attributed to error in the assumption of an initial fat content of 24%. An initial fat content of 20%, for instance, gives final compositions of the same order as with the 24% figure. The absorption of the mussel's and herring's fats by the eels should be almost complete. McCay & Tunison [1934], in experiments on trout, showed good absorption of various fats fed even at 25% of the diet. The conclusion of the writer is that there cannot have been a quantitative turnover between ingested fat and depot fat.

The hypothesis of Schoenheimer & Rittenberg [1935] may be open to question. If admixture with the fat already in the depots is thorough before withdrawal of mixed fat for combustion, then in the case of mice fed at the 1% level for 4 days one would expect to find about 95% of the dietary fat in the depots instead of the 47% actually found. It is possible, of course, that the incorporation of ingested fat in the depots is not uniform, and that the recently deposited fat is more readily withdrawn than the rest of the depot fat. Either of these conditions (i.e. partial deposition only or uneven admixture and withdrawal), or perhaps both together, could account for the observed results on eels not being fully quantitative.

An interesting point is the high fat content of eels in batch 2, Table I. Although no records of weight were taken, there was visible evidence of growth, and this on a diet of mussels. The reason is obscure, but it may be noted that the final fat showed no evidence of having been modified by the diet. The eels feeding on mussels in Exp. 2 failed to maintain weight.

Although the idea of a complete interchange between ingested fat and depot fat is not in harmony with the evidence, neither is the idea that there has been no interchange, and that the only herring's fat deposited is that represented by the total gain in fat on the diet of herring. On this basis the final fat of sample 3 (second experiment) should have consisted of entirely unchanged eel's fat.

Evidently the quantitative effect of the dietary fat on the depot fat is governed by factors which are still obscure. The metabolic rate appears to have something to do with it, since the warm-water fish, in spite of ingesting more herring's fat than did the cold-water fish, did not modify their fat so much.

Temperature. In considering the effects of temperature we should not compare various samples with the control estuarine sample, since the temperature

of the estuary was variable and unknown. In Table II we can compare sample 2 with samples 3 and 4, where the only difference was one of 9° in temperature. There is a slight suggestion that the fat in cold water is more unsaturated (certainly not shown by the iodine value, however, as seen from Table I). C_{16}, C_{20} and C_{22} unsaturated acids are slightly more unsaturated in sample 2, whilst C_{18} acids are about the same. There is evidence of the production of a more saturated fat at higher temperatures, but the effect is certainly not a large one.

In the second experiment we may compare samples 3 and 4, with similar results. C_{16}, C_{18} and C_{22} acids are slightly more unsaturated in the cold water than in the warm water, C_{20} acids being about the same. In this experiment, however, the position is complicated by the different amounts of herring's fat which have been incorporated in the depots. Herring's fat is more unsaturated than eel's fat for C_{16} and C_{18} acids, and less unsaturated for C_{22} acids. C_{20} acids are about the same (if both samples of estuarine eel's fat are considered). The fact that less herring's fat has been incorporated in the fat of the eels in sample 5 than in that of the eels of sample 4 would mean that the results for C_{16} and C_{18} acids could be accounted for on this basis. The results for C_{22} acids could not, however, be so interpreted and so here the evidence supports that of Exp. 1.

Actually, whilst as far as total groups are concerned the herring's fat appears to be deposited with the relative proportions unchanged, the degrees of average unsaturation show distinct changes. In Table VII are given the degrees of average unsaturation of C_{16}, C_{18} and C_{22} unsaturated acid groups.

Table VII. *Degrees of unsaturation of eel fats*

	Degrees of unsaturation		
Material	C_{16}	C_{18}	C_{22}
62% eel fat 38% herring fat	−2·7 H	−3·3 H	−7·5 H
Sample 3	−2·4 H	−3·0 H	−7·5 H
78% eel fat 22% herring fat	−2·5 H	−3·2 H	−8·5 H
Sample 4	−2·2 H	−2·8 H	−7·0 H

It can be seen from Table VII that the eels in both warm and cold water have partially hydrogenated either the herring's fat itself or more probably the mixed fat in the depots. In the cold water such hydrogenation has been confined to the C_{16} and C_{18} acids—the ones which the diet would tend to make more unsaturated. However, in the warm water the C_{22} acids are also hydrogenated, and this difference is presumably due to the higher temperature. The extent to which the other acids are hydrogenated is almost the same in the two samples.

Both experiments, therefore, afford evidence of greater unsaturation in fats laid down at lower temperatures, but the effect is certainly not a marked one. It is notable that whilst in both samples 3 and 4 hydrogenation has gone far enough to bring the unsaturation of the C_{16} and C_{18} acids almost to normal (Table V), there is not hydrogenation to such an extent that the iodine value of the entire fat is normal (Table IV). This is a further illustration of the comparative unimportance of the iodine value as a guide to metabolic processes in fats.

The principal effect of a higher temperature in Exp. 2 has been to accelerate the process of modifying ingested fat.

Salinity. From the results of Exp. 1 it is evident that under such conditions salinity has had no effect. Comparing samples 5 and 6 with 3 and 4 (Table II), there is no evidence at all of a tendency towards a marine type of fat in sea water or a more truly fresh-water type in the fresh water. The differences between these types have been illustrated in detail [Lovern, 1937], and consist of higher proportions of C_{16} and especially C_{18} acids, and lower proportions of C_{20} and especially C_{22} acids, in the fats of fresh-water fish. The estuarine eels' fats (controls) correspond fairly well to the fresh-water type, but are not really typical—in particular the C_{16} acids content is on the low side and the C_{20} acids content on the high side. The reason may be that they were obtained from brackish estuarine waters. That such a habitat may lead to a modified fat was suggested by the author [Lovern, 1937]. Shorland & McIntosh [1936] found that a closely related eel from New Zealand had fat resembling that of typical fresh-water fish.

It should perhaps be noted that in these experiments aeration would probably not be as complete as under natural conditions. If the differences in metabolic rate observed by Fox & Simmonds [1932] are due to the greater oxygen content of fresh water compared with sea water (as they suggest), such differences might not obtain in the experimental tanks.

It seems doubtful whether any fat was laid down under the conditions of Exp. 1 (apart from batch 2). It might be argued that the failure of salinity to have any effect was due to this, and that only fat actually deposited whilst the fish are in a certain environment can be affected by that environment. It has been shown [Lovern, 1934] that starving salmon in fresh water do not modify their fat in the direction of the fresh-water type. These eels were not starving, however, and unless the depot fat remained in a static condition with no ebb and flow (and the experiments on feeding herring definitely indicate an ebb and flow), it seems hard to avoid the conclusion that salinity has little if any direct effect.

The experiments with a diet of herring are difficult to interpret. Herring's fat is markedly marine in type, and yet 6 months' feeding on this fat at as high a level as is ever likely to be encountered in nature, did not make the eel's fat truly marine in type. It is notable that the more intensive feeding in the warm water, so far from causing the fat to become still more marine, actually resulted in less change. This suggests that there is in the animal a mechanism which can deal with ingested fat so as to modify it to the normal requirements of its depot fat. This mechanism might be unable to deal with fat above a certain level of ingestion, but its activity might well be increased at higher temperatures.

It is impossible to tell from these experiments how far it would be possible to modify the fat of a fish by an unusual diet if that diet was fed throughout its life. On the other hand, the existence of a mechanism which can modify ingested fat to a certain extent, and the fact that the rate at which this mechanism functions can be altered (e.g. by changes of temperature) is significant. Such a mechanism, with a variable rate, has been adduced for the herring [Lovern, 1938].

Summary

Using eels (*Anguilla vulgaris*) as experimental animals, an attempt has been made to determine the separate effects of diet, temperature and salinity on the composition of the depot fats of fish.

On a diet low in fat (1·1 %), the ingested fat had no detectable effect on the depot fat. On a diet high in fat (20·7 %), the eel's depot fat was appreciably modified, but the quantitative relationship between the amount ingested and the effect produced was obscure. Such ingested fat as was incorporated in the depot

fat was apparently deposited with the relative proportions of the various acids practically unchanged. Hydrogenation, however, had taken place. The eel possesses a mechanism which can modify ingested fat to a considerable extent, and the action of this mechanism is accelerated by rise of temperature.

In addition to the above property, temperature has a direct effect on the composition of fat. Lower temperatures lead to more unsaturated fat and higher temperatures to increased saturation. This effect is relatively a small one, and in natural conditions cannot be important.

The effect of salinity has not been thoroughly settled, but with a low intake of fat it appears to have no direct significance.

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CLXIV. ON THE RESPIRATORY METABOLISM OF *HELIX POMATIA*

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THE results brought forward in this paper have been accumulated during the last three years in the course of experiments on the respiratory metabolism of the edible snail, *Helix pomatia*. This paper does not purport to give a complete picture of the mechanisms concerned in the metabolism of this animal, but rather to summarize the situation as it appears at present, to indicate some metabolic peculiarities which have come to light and to draw attention to some interesting problems which still await solution. The work arose out of an attempt to obtain some information as to the general metabolism of carbohydrates in *Helix*, and it seemed worth while to extend the experiments to cover some other substrates of oxidation.

MATERIAL AND METHODS

The tissue used was the so-called "liver" or hepatopancreas of *Helix pomatia*, an organ which, according to Biedermann [1911], is responsible for the digestion, absorption and storage of the food. There can be little doubt of the metabolic importance of this organ. It is a large, dark brown structure which accounts for the greater part of the whole visceral mass, and produces numerous enzymes, including cellulase, α- and β-glucosidases, α- and β-galactosidases, fructosaccharase and other but less well-defined carbohydrases such as lichenase [see Krüger, 1933; Vonk, 1937]. Proteinases and aminopolypeptidase [Rosèn, 1934] and one or more lipases [Kuntara, 1934] have also been found either in the secretion or in the substance of the gland itself.

The main stock of animals was kept in the refrigerator and samples of a dozen or so were taken out often enough to keep up a supply of experimental animals. The use of manometric technique and tissue slices were the principal methods employed, the directions of Dixon [1934] being followed with only minor alterations. Slices were cut from the hepatopancreas of animals which had been starved for 2 to 4 weeks at room temperature; this preliminary starvation went far towards removing stored foodstuffs and made it possible to cut thin slices from the tissue which, when full of stored materials, has the consistency of a soft rubber sponge and is exceedingly difficult to manipulate. The slices were washed in the phosphate-buffered saline described below (agitated by an air blast) till the washings were no longer turbid. No definite indications of a loss of coenzymes or carriers were encountered at any stage in this work.

After the slices were freed from surplus fluid by draining on filter paper, they were weighed on the torsion balance before being placed in the manometric flasks. The dry wt. was determined on a separate small sample of the same tissue when sufficient material was available. In all, 47 such determinations were carried out, the results varying from 14 to 22 % and averaging 18 % of the wet wt. This value was assumed in cases where a separate estimation of the dry wt. was not made.

The solution used for washing and suspending the tissue slices had the composition given below. The various substances the effect of which was to be tested were added to the stock solution, usually to give a final concentration of $M/100$:

	g./l.
$Na_2HPO_4, 12H_2O$	16·120
NaH_2PO_4, H_2O	0·690
NaCl	0·141
KCl	0·443

This solution is allowed to stand for at least 3 days before use since, for some reason as yet undetermined, the use of perfectly fresh preparations occasionally leads to atypical results. The medium contains $M/20$ phosphate, a concentration which lies between the $M/10$ solution used by Dixon & Elliott [1929] and which van Heyningen [1935] believes to be detrimental to animal tissues, and the $M/40$ solution which van Heyningen himself recommends. It was found necessary to use a fairly high concentration of buffer since the tissue itself has a distinctly acid reaction, and as much as 500 mg. of it had sometimes to be suspended in only a few ml. solution. The depression of the freezing point (0·31°), the *p*H (7·73 at 28°, electrometric), and the ratio Na^+/K^+ of this medium are the same as those of the haemolymph of normal active snails [see Baldwin & Needham, 1934].

The O_2 uptake of slices suspended in this solution fell off slowly with time, but the same thing happens in CO_2-$NaHCO_3$ Ringer [Baldwin & Needham, 1934], and seems to be due to fragmentation of the somewhat fragile tissue rather than to any deleterious effect of the suspension medium. Every care was taken to minimize fragmentation by keeping the shaking machine well lubricated and reducing the rate of shaking to about 70 oscillations per min. As a rule readings were taken only over the first ½ hr., and very seldom over more than 1 hr., and during this period the respiration remained practically linear in nearly every experiment.

The "direct" technique described by Dixon [1934] was used for measuring the rate of respiration, the flasks being filled with pure O_2 and shaken in an electrically controlled bath maintained at 28°. This temperature has been repeatedly used for molluscan tissues and seems perfectly satisfactory [see for example Baldwin, 1935]. Where other methods have been employed they will be referred to in the text.

Results

It was found that there is considerable variation in O_2 consumption from one hepatopancreas to the next, and this made it necessary that all the slices used in any given experiment should be prepared from one and the same animal. Since it is often impossible to cut uniform slices of the tissue, and as the organ is too small to allow of the preparation of a large number of slices from which the best could be selected, the measurements were usually made in duplicate. The results then showed a mean variation of the order of 10%, and consequently each experiment was repeated several times and the results averaged. Each experiment mentioned in the protocols therefore refers to duplicate determinations on one and the same individual except in a very small proportion of cases. The rates of O_2 uptake are expressed throughout the paper in the usual Q_{O_2} notation, with reference to *p*H 7·7 and 28°. The quotients found for the control and experimental samples are represented by Q^c and Q^e respectively as a matter of convenience.

The effect of saccharides and other substances upon respiration

It has long been known that large quantities of glycogen can be stored in the hepatopancreas and that it disappears during starvation [von Fürth, 1903; Biedermann, 1911]. Probably much is used in muscular activity, for recent advances in muscle chemistry indicate that all muscles probably make use of closely similar mechanisms for their energy production. Very little is known about carbohydrate metabolism in *Helix*, although this form has been studied more than any other gastropod type. Schwartz [1935] and Wolf-Heidegger [1935] studied the effects of injecting adrenaline and insulin into snails, but could find no significant changes in the blood sugar level. Ermakov *et al.* [1935] on the other hand find that persistent hyperglycaemia develops after extirpation of the peripharyngeal ganglia or the injection of nicotine, while transient effects follow piqûre of the ganglia. These workers are of the opinion that the blood sugar is under nervous control.

In addition to glycogen a second polysaccharide, galactogen, is present in the snail. This substance, to which we must refer again, has been studied by May [1931–34], May & Kordowich [1932], and Schlubach & Loop [1937]. Investigations which began before the paper of Schlubach & Loop appeared are also in progress in this laboratory; the results lead us to believe that from the purely chemical point of view May's conclusions are not entirely reliable, and we hope to have definite evidence regarding this in the near future. In the most recent of his papers May has brought together the pertinent observations of earlier workers, and comes to the conclusion that galactogen is a polysaccharide consisting entirely of galactose units and having a peculiar importance in reproductive processes. There are indications in the work of Levene [1925] upon the slimy secretions of *H. aspersa* and *H. pomatia* that a polygalactose or perhaps an acetylated polygalactose may also be important in connexion with slime formation. May has shown that whereas glycogen is present in practically every part of the body of *Helix*, only the albumin gland contains galactogen, and here glycogen is absent. Galactogen accumulates in the albumin gland during the period prior to egg laying and is formed in quantities larger than can be accounted for by the amounts of galactose ingested, presumably being formed from some other sugar, such as glucose [May, 1934, 2]. The eggs themselves are remarkably rich in galactogen and contain no glycogen [May, 1932, 2], but if the animals are starved galactogen may disappear again and be utilized by the organism, but no eggs are then laid [May, 1934, 4]. It is likely therefore that glycogen (or glucose) and galactogen (or galactose) are interconvertible in the organism.

It should be mentioned in passing that one of the two samples of galactogen used in the present work was prepared from the albumin glands of a number of snails. The glands were crushed in alcohol and allowed to harden, boiled with 30% KOH, precipitated with alcohol several times and finally purified by precipitation with alkaline $CuSO_4$ [May, 1934, 1], followed by repeated precipitation with alcohol. The other sample, kindly given to me by Dr N. W. Pirie, was obtained from the whole bodies in a similar way and freed from glycogen by treatment with salivary amylase. Both preparations were free from reducing sugars and contained over 90% of galactogen.

The carbohydrates studied were added to the tissue in the form of a 2·5% solution, the final concentration in the flask being *c.* 0·25%. The results are summarized in Table I. It will be seen that most of the sugars added were without effect, since changes of less than 10% cannot be regarded as significant on the basis of only a small number of experiments. Apart from the results

Table I. *Effect of carbohydrates on respiration of snail hepatopancreas*

Carbohydrate	Averages Q^c	Averages Q^e	Increase %
Glycogen (5)	3·44	3·35	− 3
Galactogen (4)	3·51	4·68	+33
Inulin (3)	3·62	3·49	− 4
Raffinose (2)	2·54	2·63	+ 4
Maltose (2)	2·85	2·62	− 8
Lactose (2)	3·00	2·89	− 4
Sucrose (2)	4·42	4·11	− 7
Fructosediphosphate (2)	3·48	3·31	− 5
Glucose (5)	2·93	3·01	+ 3
Mannose (4)	3·76	3·87	+ 3
Galactose (10)	2·66	3·85	+44
Fructose (4)	3·89	4·64	+19
Xylose (6)	2·93	3·42	+17
Arabinose (3)	3·00	2·94	− 2

Note: The figures in brackets refer to the number of experiments carried out with each substrate. *None of the experiments was carried out during the "winter period" (Dec.–Feb.)*; experiments performed at this time are considered separately.

obtained with galactose and galactogen, the general picture is not unlike that given by mammalian liver. Fructose and xylose cause small increases in the rate of respiration, but glucose, glycogen and fructosediphosphate are not oxidized. Correlated with this there is a very low rate of anaerobic glycolysis; a $Q_M^{N_2}$ value of about 1 was found at 28°, and Chapheau [1932] has found results of the same order (0·8–1·0) for the hepatopancreas of another mollusc, the oyster. These figures are comparable with those of 2–3 at 38° recorded for mammalian livers [see Krebs, 1933, 1]. The rates of respiration too are of the same order as for mammalian liver, for the Q_{O_2} values found in all the 92 control samples quoted in this paper (i.e. those to which no substrate was added) varied from −1·13 to −5·19 at 28°, with an average of −2·93, which is very like Chapheau's figure of *c.* −2 at 28° for the oyster hepatopancreas, and may be compared with the values of −3·4 to −18·7 for mammalian liver at 38° [Krebs, 1933, 1]. There is thus a general qualitative and quantitative resemblance between the activities of the molluscan hepatopancreas and the mammalian liver. Dorman's [1928] contention, that the invertebrate "hepatopancreas" and the vertebrate liver are homologous, could thus claim a certain amount of biochemical support.

But the parallel breaks down when we consider the large effects produced by galactose and galactogen which contrast interestingly with those of glucose and glycogen. It is possible that galactogen, which has a smaller effect than its parent sugar, may be converted into galactose before being oxidized. Neither galactosemonophosphate nor glucosemonophosphate has yet been tried, but it seems unlikely in view of the other results that the latter would be oxidized. Although the galactose ester has been shown not to be attacked by a yeast trained to act on galactose [Grant, 1935], it might be attacked here if phosphoric esters play any part in the suggested conversion of galactogen into galactose. The existence of an enzyme which catalyses the oxidation of galactose is very interesting since it seems that, in general, galactose is converted into glucose before being metabolized [see Grant, 1935]. The presence of such an enzyme in *Helix* must presumably be correlated with the fact that galactose is a major metabolite in this species.

Attempts to demonstrate the presence of dehydrogenase systems with the aid of the methylene blue technique were wholly unsuccessful. Whether the tissue was used in the form of a brei or as slices, the reduction time ran into many

hours in most cases, even in the presence of added substrates. It was therefore necessary to have recourse to manometric measurements again, and the effects of pyruvate, lactate, succinate and α-glycerophosphate have been studied. As Table II shows, only the last three of these substances produced considerable

Table II. *Effect of various substances on the rate of respiration of snail hepatopancreas*

Substrate (M/100)	Averages Q^e	Averages Q^e	Increase %
Pyruvate (3)	2·02	1·86	− 8
Lactate (3 + 5*)	2·51	3·04	+21
Succinate (3 + 4*)	2·80	4·00	+43
α-Glycerophosphate (7*)	2·33	3·32	+43
Alanine (3*)	3·26	4·16	+27

Note: The figures in brackets refer to the number of experiments carried out. Where the figure is marked with an asterisk the corresponding number of experiments was carried out during the "winter period".

increases in the rate of respiration. That pyruvic acid is without effect indicates that it is not converted by the tissue into succinic acid at any considerable rate, though this transformation is known to take place in moulds [Butkewitsch & Fedoroff, 1929] and in yeast [Wieland & Sonderhoff, 1932]. The transformation also occurs in mammalian kidney, but not in liver [Elliott, 1937].

In one experiment in which the Thunberg technique was used, 500 mg. fresh slices were set up with buffer, M/100 succinate and 2 ml. 1/20,000 methylene blue at 28°, and no less than 80 min. were required for decoloration of the dye. Nevertheless, as we have seen, the rate of O_2 uptake of the slices is of the same order as for slices of mammalian liver. From the data of the experiment just quoted a Q value of about −0·03 can be calculated to correspond to the rate of reduction of the methylene blue, whereas the Q of normal respiration is about −3. O_2 is therefore reduced about 100 times as fast as methylene blue. In the case of yeast [Wieland & Claren, 1932] there is a factor of about 10, and the slowness of methylene blue reduction is due to the failure of the dye to penetrate into the cells. Methylene blue, indeed, is used as a diagnostic reagent for determining the viability of yeast cells. If these cells are disintegrated and their contents thereby made accessible to the dye, a rapid reduction begins at once. Similarly, mammalian liver reduces methylene blue more rapidly in brei than in slice form, but in the case of the snail tissue no more rapid reduction was obtained by using brei preparations.

It seemed worth while to test the hepatopancreas for the presence of a possible inhibitory substance, and this was done by comparing the rates of reduction of methylene blue by washed, chopped frog muscle in the presence of succinate, with and without the addition of aqueous extracts of hepatopancreas, but the reduction times were identical within the limits of experimental error. Although methylene blue is known to inhibit most dehydrogenases to a greater or less extent [Meldrum, 1934, p. 39], it seems unlikely that inactivation of this kind could account for the large difference found here.

The addition of methylene blue at concentrations up to 1/5000 had but little effect on the O_2 uptake of surviving hepatopancreas slices, and cresyl blue was not much more active in this respect (Table III). The mean increase found was of the order of only 20 %, which is very different from the admittedly exceptional increases of 20–30 times found by Barron [1929] for the respiration of red blood cells. Now the addition of an auxiliary O_2 carrier such as methylene blue can

only bring about an increase in the rate of O_2 absorption of a tissue if the rate of action of the reducing systems of that tissue is limited by that of the normal O_2-carrying systems, suggesting that in the present case the rate of O_2 transport in the tissue is not the limiting factor. This possibility was investigated by reducing the rate of respiration to about one-fifth by poisoning with $5.10^{-3}M$ cyanide. The experiments showed conclusively that even then the respiration is not stimulated to any significant extent (+8%) by the addition of 1/5000 methylene blue. Dixon & Keilin [1936] have pointed out, however, that the inhibition produced by cyanide is not reversible under all conditions, but it is very improbable that irreversible inhibition can have played a significant part here since, in experiments in which no precautions were taken to prevent the distillation of HCN from the suspension medium into the alkali used for CO_2 absorption, the inhibition fell off very markedly in the course of time.

Table III. *Effects of dyestuffs on respiration of hepatopancreas*

Dye and concentration	Substrate	Averages Q^c	Averages Q^e	Increase %
1/50,000 methylene blue	Galactose (3)	2·88	2·95	+ 2
1/5000 methylene blue	Succinate (3)	3·60	4·22	+17
1/5000 cresyl blue	Succinate (3)	3·04	3·93	+29

(Concentration of substrate $M/100$ in all cases.)

The failure of methylene blue to exert its usual effect when added to hepatopancreas tissue need not, however, be taken as conclusive evidence that the mechanisms of cellular respiration here are essentially different from those found in other tissues. It must be supposed that methylene blue fails to reach the reducing systems for some reason, though not, apparently, because it fails to penetrate into the cells. As we shall see, there is reason to think that the oxidation of succinic acid, at any rate, is catalysed by a typical succinic dehydrogenase in this as it is in other tissues.

A complete survey of the individual dehydrogenases has not been attempted. The data of Table II suggest that powerful succinic and α-glycerophosphate dehydrogenases are present, together with a rather weaker lactic enzyme, while those of Table I may indicate the presence of a powerful dehydrogenase for galactose. In view of the recent work of Szent-Györgyi [1937] and Krebs & Johnstone [1937], for example, it seemed desirable to carry the study of the supposed succinic dehydrogenase a little further. This was done by studying the influence upon the respiration of surviving hepatopancreas slices in the presence of succinate of (*a*) narcotics, which inhibit the majority of dehydrogenases, and of (*b*) malonate, which inhibits the succinic enzyme competitively and rather specifically.

The concentrations of various narcotics required to reduce to 50% the O_2 uptake of washed frog muscle in the presence of succinate have been determined by Sen [1931], and in the present case four different narcotics were used at the concentrations given by this author. Well washed slices of the tissue were suspended in the usual phosphate solution in the presence of $M/100$ succinate, with and without the addition of phenylurea, symm. diethylurea, urethane and phenylurethane. The data in Table IV (*a*) show that, although different individual narcotics produce varying degrees of inhibition, the average of all the results is very close to the expected 50% inhibition, and it therefore seems reasonably likely that the hepatopancreas oxidizes added succinate by means of a typical succinic dehydrogenase. More conclusive results were obtained

Table IV. *Effects of certain respiratory inhibitors*

Inhibitor	Molarity	Averages Q^c	Averages Q^e	Increase %	
(*a*) Phenylurea (2)	0·028	4·71	2·78	−41	Av. 46 %
Diethylurea (2)	0·350	3·67	1·79	−51	
Urethane (2)	0·670	3·67	1·02	−72	
Phenylurethane (2)	0·003	3·53	2·79	−21	
(*b*) Malonate (1)	0·010	4·31	4·08	−5	
,,	0·020	4·41	3·54	−20	
,,	0·050	4·49	3·11	−31	
,,	0·100	4·52	2·40	−47	
(*c*) Malonate (3)*	0·100	2·09	1·61	−23	

Substrate $M/100$ succinate in all cases except that marked with an asterisk, in which no substrate was added.

in experiments in which malonate was used. Of the respiration taking place in the presence of succinate, nearly 50 % is due to the addition of that substance (Table II), and about the same percentage is inhibited by $M/10$ malonate (Table IV (*b*)). Quastel & Wooldridge [1928] found that the addition of $M/14$ malonate inhibits more than 95 % of the activity of the succinic dehydrogenase of *Bact. coli* in the presence of $M/140$ succinate. The fact that the relative concentrations of substrate and inhibitor are the same in both these cases very strongly suggests that the respective dehydrogenases must also be similar. The effects of $M/10$ malonate upon the basal respiration of hepatopancreas slices was also tested (Table IV (*c*)) and it was found that, even in the absence of added succinate, 20–25 % of the respiration is inhibited, suggesting that the succinic dehydrogenase system may be in some way concerned in a considerable proportion of the basal respiration of this tissue.

Metabolism during the winter period

It was found in the course of experiments carried out between December 1936 and February 1937 that the usual effect of galactose upon the rate of respiration cannot in general be observed at this time of year. The more or less complete disappearance of this effect during what might be called the "winter period" is interesting from several points of view. Table V (*a*) gives a series of confirmatory data obtained during the winter period of 1937–38, and it will be observed that although a fairly, though not typically, large increase occasionally follows the addition of galactose to the tissue slices (Exps. 146, 148), the effect is absent in most cases. These data may be compared with those obtained at other times of year and shown in Table IV (*b*). The average increase for the whole series shown in Table V (*a*) is only 10 %, a value which is just on the borderline of significance. There is no reason to suppose that the winter period is associated with any peculiarities of metabolism so far as the other systems which were studied are concerned, for all these were found still to be active (Table VI). This suggests that the suppression of the galactose effect is not attributable to a breakdown on the part of the O_2-carrying system (i.e. the cytochrome + cytochrome oxidase system or its counterpart). The breakdown of the mechanisms by which galactose is ordinarily oxidized might be due (*a*) to the absence of some special carrier or coenzyme, or (*b*) to the absence of the enzyme itself. The first of these possibilities has not been tested since it was not found possible to extract any of the enzymes from the tissue itself.

Table V. *Effect of galactose on respiration of snail hepatopancreas*

Exp. no.	Q^c	Q^g	Increase %
	(*a*) During winter		
143	4·24	4·20	−1
144	2·42	2·50	3
145	1·76	1·85	5
146	1·97	2·78	41
147	1·88	2·06	10
148	1·13	1·35	19
149	2·32	2·52	9
150	2·23	2·54	14
151	1·58	1·70	8
Averages	2·17	2·39	10
	(*b*) At other periods		
8	3·85	5·31	38
15	2·51	2·62	4
19	2·40	3·52	47
22	2·18	4·06	86
30	3·38	5·92	75
64	2·22	3·10	40
65	3·22	4·79	49
66	2·45	2·91	19
152	2·45	3·33	36
153	1·95	2·93	50
Averages	2·66	3·85	44

Table VI. *Metabolism during winter period*

Substrate	Mean increases % "Summer"	Mean increases % "Winter"	Data in table no.
Galactose	44	10	V (*a*, *b*)
Alanine	—	27	II
Succinate	52	38	II
Lactate	19	23	II
α-Glycerophosphate	—	43	II

The alternative view involves consideration of the galactose enzyme as an "adaptive" one, disappearing during the winter for some reason as yet unknown, and reappearing in the spring, perhaps in response to the presence of galactose in the food which is then ingested. There is now a fair body of evidence to show that bacteria can develop enzymes of this kind in response to the presence of certain particular substances in the culture media, and even among Metazoa there are examples of the production of adaptive enzymes [Stephenson, 1937; Yudkin, 1938].

It seems possible that the suppression of the oxidation of galactose by the hepatopancreas during the winter period may be a physiological, seasonal change, correlated with the fact that the snail normally spends this part of the year in hibernation. It is not known whether the seasonal changes in metabolism which take place in certain animals are associated with corresponding "adaptive" changes in enzymic activity, even in the extreme cases in which hibernation takes place. Indeed, we know very little about the metabolism of hibernation [Gorer, 1930]. It is generally believed that in the cases of the hedgehog and the marmot, the metabolism of carbohydrate is almost entirely suppressed in favour of that of fat during the winter sleep, but whether there are any changes in the enzymes concerned we cannot say. Considerable metabolic differences exist between winter and summer frogs [see Tigerstedt, 1914, and Adler, 1921, for example], and seasonal variations are known even in animals which do not pass

the winter in a state of inanition. Sherwood [1936], for instance, has found small but perfectly definite seasonal changes in the basal metabolic rate of rats. Seasonal variations such as these may well be associated with changes in enzymic activity, and the disappearance, or at any rate the suppression, of the oxidation of galactose in "winter" snails may perhaps be an example of this.

There is reason to believe, however, that galactose can be utilized in some way by winter snails, for galactogen may disappear from the albumin glands of starved specimens after the stores of glycogen have been depleted [May, 1934, 4]. If, as seems to be the case, we must regard galactogen as a substance which is stored up in preparation for the egg-laying season, suppression of the oxidation of galactose during hibernation might be regarded as a device for conserving this polysaccharide as far as possible.

Oxidase systems

The presence of xanthine oxidase in the hepatopancreas of *Helix* has already been demonstrated by Przyłecki [1926] and confirmed by Baldwin & Needham [1934], but it could not be shown by the technique used in the present experiments. No increase in the rate of respiration (-2%) could be found following the addition of $M/100$ hypoxanthine to tissue slices, or to a preparation obtained by grinding the fresh tissue with half its own weight of sand and five volumes of phosphate buffer, centrifuging, half-saturating the centrifugate with ammonium sulphate, centrifuging again and finally taking up the precipitate in phosphate buffer. It is known, however, that xanthine oxidase has a great affinity for its substrate. For the xanthine oxidase of milk, Dixon & Thurlow [1924] give a Michaelis constant of $3.10^{-5}M$, and it is quite possible that in the present experiments the enzyme was already saturated with its substrates, considerable amounts of which are believed to be present in the hepatopancreas tissue [Wolf, 1933].

The presence of amino-acid deaminase was readily demonstrated. Since pyruvate had already been found to have no effect upon the rate of respiration, alanine was a suitable substrate for these experiments, and a $M/100$ solution of the *dl*-acid was employed. The results are shown in Table II. The presence of the deaminase was also detected by shaking 5·23 g. washed slices with 500 mg. alanine in 25 ml. CO_2-$NaHCO_3$ Ringer [see Baldwin & Needham, 1934] to which 1 ml. saturated As_2O_3 had been added [cf. Krebs, 1933, 2]. The shaking was continued for 5 hr. at 28° and the fluid then deproteinized with 5 ml. 30% trichloroacetic acid. When cold, the filtrate received 10 ml. saturated 2:4-dinitrophenylhydrazine in $2N$ HCl, and crystallization began at once. The whole was left in the refrigerator till next day and the crystals were then collected on the centrifuge, washed twice with $N/4$ HCl and three times with distilled water and finally dried *in vacuo*: M.P. 208°. After recrystallization from ethyl acetate the purified product came out in hexagonal plates, M.P. 216°. Found: C, 40·4; H, 3·1; N, 21·0%. Calc. for the 2:4-dinitrophenylhydrazone of pyruvic acid; C, 40·3; H, 2·99; N, 20·9%. An entirely similar product was obtained by allowing an acetone powder preparation of the hepatopancreas to act upon alanine [cf. Krebs, 1935].

Colour tests were carried out on *p*-phenylenediamine, *o*-phenylenediamine, catechol, 3:4-dihydroxyphenylalanine and tyrosine. When well washed slices of the hepatopancreas were added to dilute aqueous solutions of these substances a deep purple coloration was produced within ½ hr. in the case of *p*-phenylenediamine, but the other reagents showed no appreciable colour change after 24 hr.

If the slices were not thoroughly washed, weak positive reactions were given also by catechol and 3:4-dihydroxyphenylalanine, and Pugh [1930] has found that these substances, but not monohydric phenols, are also oxidized by extracts prepared from the large black slug, *Arion ater*. In both these cases tyrosinase must be regarded as absent. But it seems difficult to attribute the powerful and decidedly specific oxidation of *p*-phenylenediamine to any enzyme other than the cytochrome (indophenol) oxidase.

Confirmation of the presence of this enzyme was obtained manometrically by measuring the increase in O_2 uptake resulting from the addition of a few mg. solid *p*-phenylenediamine to slices of hepatopancreas respiring in $M/100$ succinate. Characteristically large increases (average +360 %) followed the addition of this reagent. The effect was counteracted to the extent of 70–80 % by the simultaneous addition of $10^{-2}M$ cyanide, and the presence of cytochrome oxidase thus seemed to be very probable. Further confirmation of this was obtained by taking advantage of the fact that cytochrome oxidase is powerfully inhibited by cyanide. In the majority of animal tissues it seems that 60–80 % of the total respiration is inhibited by $10^{-3}M$ cyanide and therefore mainly due to the cytochrome system.

The results of much early work on cyanide inhibition were invalidated because no precautions were taken to prevent distillation of cyanide from the suspension medium into the alkali used for CO_2 absorption, but Krebs [1935] has described equilibrating solutions which overcome this difficulty. These were used in the present experiments, and with very satisfactory results, for whereas the inhibition passed off with time if this precaution was not taken, no such effect was detectable when Krebs's solutions were employed. The results of the experiments, which were carried out in the presence of $M/100$ succinate, are shown in Fig. 1. They closely resemble those obtainable with many other animal

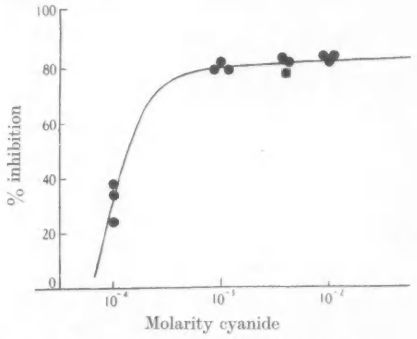

Fig. 1. Effect of cyanide on respiration of hepatopancreas.

tissues. A maximal inhibition of 80–85 % is reached at a cyanide concentration of $10^{-3}M$, and the concentration of cyanide required to inhibit 50 % of the total cyanide-sensitive respiration is about the same as that for cytochrome oxidase. It therefore seems reasonably certain that cytochrome oxidase is present in the hepatopancreas.

Perhaps there has been no more fundamental contribution to the subject of cellular respiration than the discovery by Keilin [1925] of cytochrome and of the part which it plays in living tissues [Keilin, 1929; 1933]. It seems, indeed, that

the cytochrome + oxidase system is of almost universal occurrence in aerobic organisms, but its functional importance has been definitely established in only a few particular tissues. Although various unicellular organisms and some mammalian tissues have now been examined it must be owned that the lower Metazoa have scarcely been studied at all from this point of view, although Keilin [1925] has studied the biological oxidation and reduction of cytochrome in the wing muscles of the wax-moth, *Galleria*.

An attempt was made to demonstrate the presence of cytochrome by micro-spectroscopic examination of slices of the hepatopancreas. Whereas Keilin's [1925] finding of cytochrome in the muscles of the radula was readily confirmed, no trace of cytochrome could be found in the hepatopancreas. In this respect again the hepatopancreas resembles mammalian liver. The tissue is so darkly coloured that only small thicknesses of it could be examined, and it is therefore not impossible that minute amounts of cytochrome may have been present but evaded detection. The only visible band corresponded to the α-band of reduced helicorubin, and this was visible even after the tissue, which was used in the form of thin slices piled on top of each other, had been subjected to very vigorous washing.

This pigment, helicorubin, is very widely distributed. It occurs in the gut juice of starving specimens of *Helix*, and according to MacMunn [1885], who described it under the name of enterohaematin, it is present also in the gut contents of many other gastropods and also in some crustaceans. Pantin [1932] states that it is to be found also in the gut of an annelid worm, *Aphrodite*. It appears to be made in the hepatopancreas and poured out into the gut, and "the helicorubin in the liver of the crayfish appears to be exactly the same as molluscan helicorubin" [Anson & Mirsky, 1925]. In *Helix* at any rate it must probably be of physiological importance, for if a starving snail be fed on filter paper, no trace of helicorubin can be detected in the faecal mass. This suggests that the pigment is not to be regarded simply as a waste product, analogous perhaps to the bile pigments of the mammals, but that it is actively retained in the body. In view of the fact that it is autoxidizable at the rather acid pH of the gut contents, Anson & Mirsky [1925] suggested that it might act as a respiratory pigment, but since it is found in the gut and the hepatopancreas it is a little difficult to see how it could play any important part in the respiration of the animal as a whole.

It seems not inappropriate at this point to put forward the tentative hypothesis that, while cytochrome is in all probability the chief ultimate H acceptor in many tissues, it is replaced by helicorubin in the hepatopancreas of *Helix*, and perhaps elsewhere. Experiments are in progress with a view to testing the possible truth of this hypothesis, and in the meantime there is some apposite information. It is already known that cytochrome is replaced by other haematin compounds in certain tissues, among which mammalian liver is included. Keilin [1929] has shown that its place is taken by another type of haemochromogen in facultative anaerobes, the liver cells of mammals and in the cells of some gastropods. The actiniohaematin of the Actinia is also a pigment of this kind [Roche, 1936, 2; see also the review by Roche, 1936, 1]. Helicorubin itself is a haematin pigment, the haem of which is identical with that of haemoglobin [Dhéré & Vegezzi, 1917; Anson & Mirsky, 1925] and very like that of cytochrome *c*, if not identical with it [Hill & Keilin, 1930]. In slightly acid solutions it is autoxidizable, a property which it shares only with haemoglobin, and with cytochrome in rather strongly alkaline solutions. Near the neutral point it loses this property but is then oxidizable in the presence of cytochrome oxidase,

just as is cytochrome itself under physiological conditions [Keilin, 1925, 1933]. These properties fit it, more than any other known haematin compound, to take the place of cytochrome.

Evidence for the presence of cytochrome oxidase in the hepatopancreas of *Helix* has already been presented and there can be little doubt therefore that helicorubin, which is certainly present in this tissue, can be oxidized there under the conditions obtaining *in vivo*. With regard to its possible reduction under biological conditions, some preliminary experiments have already shown that it is very rapidly reduced by traces of cysteine, and rather more slowly by certain biological systems. Further work in this direction is in progress.

Summary

1. The results of a number of experiments on the respiratory metabolism of the hepatopancreas of *Helix pomatia* show that on the whole this tissue resembles the liver tissue of vertebrates fairly closely, but differs markedly from it in that its rate of respiration is much increased by galactose and galactogen.

2. Succinate, lactate, α-glycerophosphate and alanine also increase the rate of respiration.

3. During the winter the oxidation of galactose is largely suppressed, though succinate, lactate, α-glycerophosphate and alanine still produce their usual effects. It is suggested that this may be physiological seasonal change.

4. The oxidation of succinate appears to be due to a typical succinic dehydrogenase. This is inhibited by narcotics and by malonate in the same way and to the same extent as the succinic dehydrogenase of other tissues.

5. No rapid reduction of methylene blue by the tissue could be demonstrated under any of the conditions tried, nor could the respiration of normal or cyanide-poisoned tissue be accelerated by means of methylene blue.

6. Cyanide in $M/1000$ solution inhibits 80 % of the respiration of the hepatopancreas, and the inhibition seems to be attributable to the poisoning of cytochrome oxidase. The presence of this enzyme seems to be highly probable.

7. No cytochrome could be detected spectroscopically in the tissue; the only haematin compound detectable in this way is helicorubin. A possible function of this pigment is tentatively suggested and discussed.

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CLXV. COMPOSITION OF THE MILK FROM THE BREASTS OF NEWLY-BORN INFANTS

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(*Received 26 May 1938*)

It is well known that most infants of both sexes secrete milk when newly born, the secretion being termed witches' or sorcerers' milk. Halban [1904] described the phenomenon as a puerpural involution in the mammary glands of the newly born infants. Billard [1837] considered the secretion to be imperfect milk loaded with leucocytes, often ending in abscesses. Schlossberger [1852] has given an analysis of one sample, viz. total solids 3·25, fat 0·82, protein and sugar 2·83 and ash 0·5 %. Three analyses by Gessner [1867] show the secretion to contain total solids 3·7–10·6, fat 0·8–1·5, total protein 1·1–2·8 and lactose plus ash 1·9–6·4 %.

In the present investigation, 5 samples of such secretions were collected from infants of both sexes between 9 and 17 days old and submitted to as comprehensive an analysis as the small yields obtained permitted. The milk was obtained by gentle squeezing of the enlarged breasts, the milk then being sucked up by a pipette and transferred to small glass bottles containing a trace of formalin. The yields were variable but in no case exceeded 1·8 g.

By suitable management of fractions and adoption of microchemical technique, the following analytical data were collected: total solids, nitrogen distri-

Table I. *Composition of milk from breasts of infants*

Sample No.	1	2	3	4	5
Sex	M. and F.	M.	F.	M.	F.
Age (days)	8 (M.) and 17 (F.)	9	14	10	9
Yield (g.)	1·20	0·35	0·84	1·77	0·65
As % of milk:					
Total solids	8·67	12·50	7·36	9·76	10·57
Protein (N × 6·38)	3·40	7·23	4·60	2·68	8·31
Lactose	1·80	1·56	1·56	2·67	1·00
(as glucose)	0·99	0·86	0·86	1·47	0·55
Fat + ash, etc. (by difference)	3·47	3·71	1·20	4·41	1·26
Cl	0·14	0·10	0·14	0·10	0·12
Total N	0·53	1·13	0·72	0·42	1·30
Protein N	0·34	0·62	0·51	0·28	0·71
Casein N	0·22	0·52	0·36	0·21	0·56
Non-protein N	0·19	0·51	0·21	0·14	0·59
Osmotic equivalents:					
Lactose equivalent of Cl	2·74	1·96	2·74	1·96	2·35
Lactose equivalent of N.P.N. (calc.)	2·50	4·30	2·70	2·40	3·60
As % of total N:					
Protein N	65	55	70	67	55
Casein N	40	46	49	33	43
Non-protein N	35	45	30	34	45
Casein in protein %	63	84	70	50	79
Peroxidase	+	+	+	+	+
Phosphatase (units per 0·5 ml.)	7·6	7·4	7·5	5·1	6·9

bution, sugar and chloride content and evidence of the presence of peroxidase and phosphatase. The weights of samples were unfortunately too small to determine fat and mineral distribution; the amount of ash was in all cases too small to be determined accurately.

Total solids were determined by drying at 100°. Nitrogen distributions were studied by separating the casein at *p*H 4·6 with an acetate buffer and centrifuging, and total protein was determined by precipitation with 4 % trichloroacetic acid at 70°; all the nitrogen fractions were determined by micromethods. Sugars were determined by Maclean's method for blood sugar. Peroxidase was tested for by the benzidine-H_2O_2 method and phosphatase by the Kay & Graham [1935] technique. Table I gives the results obtained for the 5 samples.

All samples appeared to contain fat since they showed the property of creaming on standing; no churning of the fat had occurred during transit in the post. The fat content was not determined.

Discussion of results

The total solids content was regularly higher than that reported by other workers, except a value for one sample given by Gessner. The highest total solids occurred around the 9th day, protein accounting for most (60–80 %) of the solids. It is probable that the secretion is at first watery, rises in total solids and protein content to the 9th day, and then decreases in solids slowly and in protein rapidly, due to resorption in the following days.

The resorption period yields a secretion which is higher in lactose and chloride than the pre-resorption period. If the secretion obeys the same osmotic laws as normal milk, it can be stated that the higher non-protein nitrogen content of the secretions containing high total nitrogen contents balances osmotically the low contents of sugar and chloride. This was roughly tested out in the following manner. In normal milk with only 6 % of the total nitrogen as non-protein nitrogen, the relationship between lactose and chloride is expressed by the formula:

$$\text{Lactose } \% + 19{\cdot}6 \text{ Cl } \% \text{ (A.)} = 7{\cdot}0 \text{ [Davies, 1932].}$$

None of the present secretions obey this relationship, owing to the high non-protein nitrogen content. The value 7·0–A. was plotted against the percentage of non-protein nitrogen for each case. This gave points which lay roughly on a straight line represented by the equation:

$$\text{Lactose equivalent of non-protein nitrogen} = \text{N.P.N. } \% \times 3 + 2.$$

Sugar, chloride and non-protein nitrogen are thus responsible for most of the osmotic pressure of these secretions.

Protein. The amount of the total true protein represented by casein was variable, ranging from 50 to 84 %. Generally, casein percentage was highest when total nitrogen was highest. The non-casein protein, according to qualitative precipitation tests, contained globulin, as saturation of casein-free filtrates with Na_2SO_4 and $MgSO_4$ gave flocculent precipitates. The amount of non-casein protein was lowest for samples with high non-protein nitrogen content.

General. The secretions bear some resemblance in composition to those from the udders of pregnant heifers and dry non-pregnant cows [Woodman & Hammond, 1923], especially the latter. Close similarity exists in the low lactose, high total protein with low proportion of casein to total protein, and high non-protein nitrogen content. The appearance of globulin in amounts above those found in normal milk in the above and in infant secretions is undoubtedly associated with the abnormal functioning of the mammary glands, such as is found

before and immediately after parturition (colostrum). The secretion in infant breasts tends to resemble colostrum in composition, the main points of difference being the very low lactose and high non-protein nitrogen which does not occur in the latter.

The secretions gave strong peroxidase reactions, due probably to the presence of leucocytes which were observed in all samples by microscopic examination. The phosphatase content was of the same level as that found in normal cow's milk.

SUMMARY

The analyses of 5 samples of secretions from the breasts of newly-born infants from 8 to 14 days old are reported. The total solids, which range from 7·4 to 12·5%, consist mostly of protein and lactose. Compared with normal milk, the secretions are low in lactose but high in globulin and non-protein nitrogen. The secretions contain appreciable amounts of peroxidase and phosphatase.

The authors are indebted to Dr S. J. Folley for his assistance and interest in the work.

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CLXVI. THE SPECIFICITY OF ANEURIN AND NICOTINAMIDE IN THE GROWTH OF *STAPH. AUREUS*

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(*Received 2 June 1938*)

It has been shown [Knight, 1937, 1, 2, 3] that nicotinamide and aneurin (or certain closely related derivatives corresponding to these two compounds) are necessary for the growth of typical strains of *Staph. aureus* in a medium of known chemical composition.

The activities of several compounds closely related to nicotinamide and aneurin which were previously tested [Knight, 1937, 3] showed that only a limited departure from these two chemical structures was compatible with the maintenance of growth activity. It was further found that a mixture of a pyrimidine and a thiazole, both having substituents corresponding to those of the component pyrimidine and thiazole rings of aneurin, could be used in place of aneurin itself. The behaviour of the further series of compounds recorded in the present paper emphasizes the specificity of both the nicotinamide and the aneurin structures in the growth requirements of *Staph. aureus*.

Technique

The methods used for the growth tests were the same as those previously described [Fildes *et al.* 1936; Fildes & Richardson, 1937]. The test organism was a typical strain of *Staph. aureus* which could not grow on the basal amino-acid medium without the further addition of nicotinamide and aneurin (or the equivalent pyrimidine + thiazole; see below).

The substances to be tested fell into four categories:

(1) Aneurin analogues: as substitutes for the complete aneurin molecule.

(2) Pyrimidine series: as substitutes for the pyrimidine component of aneurin.

(3) Thiazole series: as substitutes for the thiazole component of aneurin.

(4) Pyridine series: as substitutes for nicotinamide.

The compound to be tested was added in serial dilution to the basal medium which was nutritionally adequate in all respects except for that component, the substitute for which was under test. Thus of the complete group of nutrients required for growth only one component was varied at a time. Pyridine derivatives (as substitutes for nicotinamide) were always tested in presence of an excess of aneurin ($1 \times 10^{-7} M$). Similarly aneurin analogues or derivatives (pyrimidines and thiazoles) were tested in the presence of an excess of nicotinamide ($1 \times 10^{-6} M$). When the component parts of the aneurin molecule were being investigated separately, i.e. either the pyrimidine or the thiazole moiety

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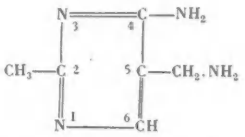
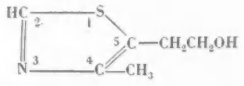
was being varied, then an excess of the corresponding thiazole or pyrimidine was added. Thus when testing thiazoles, 2-methyl-4-amino-5-aminomethylpyrimidine was used as the complementary active pyrimidine; conversely when testing pyrimidines 4-methyl-5-β-hydroxyethylthiazole was used.

Growth was aerobic and incubation was continued for 3 days to allow time for slow utilization, if it occurred, to become apparent. In general the picture of activity shown within 24 hr. was not altered, qualitatively or quantitatively, on further incubation. Only in two cases have compounds apparently inactive at 24 hr. been found to permit growth on longer incubation; these compounds were methyl nicotinate [Knight, 1937, 3 and Table IV] and 4-methyl-5-vinylthiazole (see No. 14, Table III).

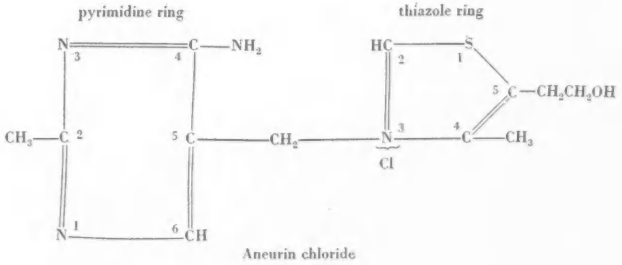
The results obtained are collected in Tables I–IV; for completeness and convenience of comparison the chief results previously obtained are also included. Fig. 1 shows the structural formulae of aneurin and its components for convenience of reference.



4-Amino-5-aminomethyl-2-methylpyrimidine



4-Methyl-5-β-hydroxyethylthiazole



Aneurin chloride

Fig. 1.

Aneurin analogues

In all this work where the typical active substances (e.g. aneurin) are of high potency it is necessary when testing closely related substances to be certain that apparent activities are not due to contamination by active impurities. In these circumstances failure to obtain growth is acceptable evidence of lack of activity, whereas an apparently lowered potency must be regarded sceptically, until it can be shown, either by repeated purification leading to no alteration of activity, or by the method of synthesis, that an active impurity is not the cause of the observed growth.

The much lowered potency of the "*iso*aneurin", about 1/10,000th of the activity of aneurin, is probably real. From the method of preparation [Andersag & Westphal, 1937, pp. 2050–1] no contamination with aneurin itself seems

Table I. *Aneurin analogues*

	Conc. *M*	Growth 24 hr.
Aneurin chloride HCl	1×10^{-8}	+ + + +
,,	2×10^{-9}	+ + +
,,	4×10^{-10}	+ +
,,	8×10^{-11}	?
*iso*Aneurin	1×10^{-5}	+ + +
,,	1×10^{-6}	?
,, + correct pyrimidine 10^{-7} *M*	1×10^{-7}	+ + +
,, + correct pyrimidine 10^{-8} *M*	1×10^{-8}	?
Chloroaneurin	1×10^{-5}	+ + +
,,	1×10^{-6}	?
,,	1×10^{-7}	0
,, + correct pyrimidine 10^{-7} *M*	1×10^{-8}	+ + + +
,, + correct pyrimidine 10^{-7} *M*	1×10^{-9}	+ + +
Aneurin minus hydroxyethyl group	1×10^{-5}	0
,, ,, ,, + correct thiazole 2×10^{-7} *M*	1×10^{-8}	+ + + +
2-Methylaneurin	1×10^{-5}	0
,, + correct thiazole $1{\cdot}0 \times 10^{-7}$ *M*	1×10^{-8}	+ + +

Aneurin: synthetic specimen (Merck).

*iso*Aneurin: having CH_3 at 6 instead of 2 in the pyrimidine. (I.G. Farbenindustrie; transmitted by Dr A. Lwoff.)

Chloroaneurin: having Cl instead of CH_3 at 2 and CH_3 at 6 in the pyrimidine. (Drs Bergel & Todd.)

Aneurin minus hydroxyethyl group: lacking the 5-β-hydroxyethyl group of the thiazole ring. (Bergel & Todd.)

2-Methylaneurin: having an additional CH_3 at 2 in the thiazole. (Bergel & Todd.)

In this and in subsequent tables:

+ + + +, + +, etc.; = number of + signs proportional to related mass of growth.
tr. = trace of growth just visible to the naked eye.
? = just detectable effect visible to naked eye.
0 = no detectable effect at concentration shown.

possible. Using the "*iso*aneurin" as a source of the thiazole only, that is, adding also 2-methyl-4-amino-5-aminomethylpyrimidine, there was an enhancement of activity, although not reaching the level expected from equivalent active pyrimidine + thiazole mixtures.

Similarly the much reduced potency of the "chloroaneurin" is probably real and not due to active impurity. Bergel & Todd [1937] reported that this compound apparently possessed some vitamin activity on one occasion, but further examination of the material failed to confirm this. The chloro compound was a potent source of the thiazole for *Staph. aureus* when tested in the presence of the correct pyrimidine.

The other two aneurin analogues tested have both altered substituents in the thiazole ring and their lack of activity is confirmed by the inactivity of the corresponding thiazoles in thiazole-pyrimidine pairs (Nos. 6 and 12, Table III). These two analogues were found by Bergel & Todd [1937] to be devoid of vitamin activity for rats.

Pyrimidines

The results with the new pyrimidines tested confirm the conclusions of the previous work [Knight, 1937, 3]. The apparent activity of 2-methyl-4-methoxy-5-aminoethylpyrimidine (No. 4, Table II) was undoubtedly due to contamination with the corresponding active 4-amino compound (No. 1, Table II). This is clear from the mode of preparation of the 4-methoxy compound [Andersag & Westphal, 1937, pp. 2046–7] which involves the separation of a 4-methoxy compound from the corresponding 4-amino compound by different solubilities

in methanol. This might easily permit sufficient contamination of the former by the latter to account for the apparent activity; contamination of less than 0·1 % would suffice.

The observed effects with all the pyrimidines tested show that the essential substituents of the ring for activity are: a CH_3 group at position 2; an NH_2 group at 4; and at position 5 a CH_3 group substituted in certain ways. Thus at 5, $—CH_2NH_2$, $—CH_2OH$ and $—CH_2NH.CSH$ permit growth; but $—CH_2CO.NH_2$ does not, nor does an unsubstituted $—CH_3$.

These results have a bearing on the question of whether *Staph. aureus* requires the complete molecule for its growth activity and whether it does in fact join an active pyrimidine and thiazole together to make aneurin when given the two components. The $—CH_2—$ at position 5 is that which forms the link between the pyrimidine and thiazole rings in aneurin. Since the results in Table II show that the nature of the groups attached to this 5-CH_2— group

Table II. *Pyrimidines*

	Conc. *M*	Growth 24 hr.
1. 2-Methyl-4-amino-5-aminomethylpyrimidine	1×10^{-8}	+ + + +
	2×10^{-9}	+ +
	4×10^{-10}	+
	8×10^{-11}	?
2. 2-Methyl-4-amino-5-hydroxymethylpyrimidine	1×10^{-8}	+ + + +
	1×10^{-9}	+ +
	1×10^{-10}	+
3. 2-Methyl-4-amino-5-thioformamidomethylpyrimidine	1×10^{-6}	+ + + +
	1×10^{-7}	+
	1×10^{-8}	0
4. 2-Methyl-4-methoxy-5-aminomethylpyrimidine	1×10^{-7}	+ +*
	2×10^{-8}	+
	4×10^{-9}	0
5. (2-Methyl-4-aminopyrimidyl (5))-acetamide	1×10^{-5}	0
6. 2-Methyl-4-hydroxy-5-aminomethylpyrimidine	2×10^{-6}	0
7. 2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine	2×10^{-6}	0
8. 2:5-Dimethyl-4-aminopyrimidine	1×10^{-5}	0
9. 2:6-Dimethyl-4-hydroxy-5-aminopyrimidine	1×10^{-5}	0
10. 2-Methyl-4-hydroxy-6-aminopyrimidine	1×10^{-5}	0
11. 2-Methyl-4-mercaptopyrimidine	1×10^{-5}	0
12. 2-Hydroxy-4-aminopyrimidine (cytosine)	2×10^{-5}	0

* The apparent activity of No. 4, 2-methyl-4-methoxy-5-aminomethylpyrimidine, was undoubtedly due to contamination with the corresponding 4-amino compound (No. 1, see p. 1243). The specimen was an I.G. Farbenindustrie product.

Nos. 1, 3, 6, 7: obtained from Drs A. R. Todd & F. Bergel.

Nos. 2, 4, 5, 9: obtained from I.G. Farbenindustrie and made by Andersag & Westphal [1937]; transmitted by Dr A. Lwoff (Institut Pasteur, Paris).

Nos. 8, 10, 11: obtained from Hoffmann-La Roche; transmitted by Dr A. Lwoff.

No. 12: synthesized by Dr G. M. Richardson [1936].

does affect the growth activity, this is evidence that the 5-CH_2— group is concerned in whatever function the pyrimidine has. The relative effects of the differently substituted 5-CH_2— group and the unsubstituted 5-CH_3 group probably indicate that the pyrimidine and thiazole are joined by *Staph. aureus*, this being possible with $—CH_2NH_2$, $—CH_2OH$, more difficult with $—CH_2NH.CSH$, but not possible with $—CH_2CO.NH_2$ or $—CH_3$. On chemical grounds the formation of the thiazolium compound is likely with the first three groupings; the corresponding 5-CH_2Br— pyrimidine is used in the Williams & Cline [1936] synthesis. It is not likely with $—CH_3$ and hydrolysis of

—$CH_2CO.NH_2$ would yield —CH_2COOH, having one carbon atom too many for the link.

Hypotheses suggesting that an active pyrimidine and thiazole are used by *Staph. aureus* separately for different reactions, or for different parts of the same process, with the corollary that aneurin itself is split into its two components seem improbable in the light of these findings. Hills [1938], who showed that aneurin or the corresponding pyrimidine and thiazole together were necessary for the normal metabolism (aerobic or anaerobic) of pyruvate by *Staph. aureus*, found that the maximal effect of adding the two components simultaneously appears slightly later than the full effect of adding aneurin in a parallel experiment, suggesting a short lag while aneurin was made by linking the components. Other reasons for believing that the complete molecule or derivatives of it, are the active substances required for essential metabolic reactions in *Staph. aureus*, are discussed later.

Thiazoles

The most interesting new features shown by the thiazoles now examined are the reduced but real potencies of the β- and γ-hydroxypropyl compounds (Nos. 9 and 10, Table III), the delayed effect with the vinyl compound (No. 14, Table III), and the activity of 4-methyl-5-β-acetoxyethylthiazole (No. 2, Table III). Together

Table III. *Thiazoles*

		Conc. *M*	Growth 24 hr.
1. 4-Methyl-5-β-hydroxyethylthiazole		5×10^{-8}	+ + + +
		1×10^{-8}	+ + + +
		2×10^{-9}	+ +
		4×10^{-10}	tr.
2. 4-Methyl-5-β-acetoxyethylthiazole		1×10^{-8}	+ + +
3. 4-Methyl-5-α-hydroxyethylthiazole		5×10^{-7}	0
4. 3-Benzyl-4-methyl-5-β-hydroxyethylthiazole*		1×10^{-6}	+ + +
		1×10^{-7}	+ +
		1×10^{-8}	tr.
5. 3-(4′(5′)-Methylimidazole)-4-methyl-5-β-hydroxyethylthiazole*		1×10^{-6}	+ + +
		1×10^{-7}	+ +
		1×10^{-8}	+
6. 2:4-Dimethyl-5-β-hydroxyethylthiazole		5×10^{-7}	0
7. 2-Hydroxy-4-methyl-5-β-acetoxyethylthiazole		1×10^{-5}	0
8. 4-Methyl-5-ethylthiazole		5×10^{-7}	0
9. 4-Methyl-5-β-hydroxypropylthiazole		5×10^{-7}	+ +
		5×10^{-8}	0
10. 4-Methyl-5-γ-hydroxypropylthiazole		5×10^{-7}	+
		5×10^{-8}	0
11. 4:5-Dimethylthiazole		1×10^{-7}	0
12. 4-Methylthiazole		1×10^{-5}	0
13. 4-Methyl-5-mercaptothiazole		1×10^{-5}	0
		22 hr.	43 hr. 90 hr.
14. 4-Methyl-5-vinylthiazole	5×10^{-7} *M*	0	tr. +
	5×10^{-8} *M*	0	0 0

* Nos. 4 and 5. The apparent activity of these two compounds was probably due to contamination with the correct thiazole (i.e. not substituted at 3) from which they would be prepared (see text, p. 1246).

Nos. 1 and 12. Synthesized by Drs A. R. Todd & F. Bergel.

Nos. 2 and 7: I.G. Farbenindustrie product [Andersag & Westphal, 1937]; transmitted by Dr Lwoff.

Nos. 4, 5, 11 and 13: Hoffmann-La Roche product; transmitted by Dr Lwoff.

Nos. 3, 6, 8, 9, 10 and 14: obtained from Dr E. R. Buchman (California Institute of Technology, Pasadena, U.S.A.).

these show that there is some, although limited, scope for variation in the structure of the thiazole which is an essential part of the growth requirements of *Staph. aureus*.

The activity of 4-methyl-5-β-acetoxyethylthiazole is not unexpected; conversion into the aneurin thiazole (5-β-hydroxyethyl) merely involves hydrolysis of the acetate. *Staph. aureus* is known to be able to hydrolyse an ester link, as in methyl nicotinate (see Table IV).

We are assured by Dr E. R. Buchman, who supplied us with the specimens of 4-methyl-5-β- and -γ-hydroxypropylthiazoles that the activities we observe are not due to traces of contaminating active impurity (the aneurin thiazole). He and his colleagues had occasion to examine this point with regard to *Phycomyces* growth activity (see below) and found that these hydroxypropyl derivatives *plus* the correct pyrimidine had activities equal to the corresponding aneurin analogues which had been recrystallized several times. The quantitative equivalence found could not therefore be explained by an active impurity in these two cases. We feel confident that tests of the corresponding aneurin analogues on *Staph. aureus* will confirm the real but much lower potency (e.g. 1/1000) of the 4-methyl-5-β- and -γ-hydroxypropylthiazoles found in the present tests. The hydroxypropyl aneurin analogues had no antineuritic activity in rats at a dose 1000 times the curative dose of aneurin (Dr E. R. Buchman; private communication).

The delayed activity of the 4-methyl-5-vinylthiazole (No. 14, Table III) is real; an active contamination would give an immediate response. It is reasonable to suppose that some of the active 5-β-hydroxyethyl compound is formed by addition of water to the $—CH=CH_2$ side chain. The effect with the vinyl compound is presumably delayed while this takes place.

The inactivity (no effect at 1 to $2\times10^{-7}M$) of the 4-methyl-5-α-hydroxyethyl-, 4-methyl-5-ethyl- and 4:5-dimethyl-thiazoles, and the lowered potency of the hydroxypropylthiazoles (an effect at $5\times10^{-7}M$) compared with the activity of the aneurin thiazole (4-methyl-5-β-hydroxyethylthiazole) which shows a detectable effect at $10^{-9}M$, illustrates the importance of the side chain at position 5.

It is also interesting that substitution of a $—CH_3$ or —OH group at position 2 in the thiazole ring (Nos. 6 and 7, Table III) inactivates an otherwise active thiazole; the same holds for a corresponding aneurin analogue (2-methylaneurin). When aneurin is oxidized to thiochrome it is the C atom at 2 in the thiazole ring which links with the nitrogen in the 4-amino group in the pyrimidine. The inactivity of these 2-substituted thiazoles suggests the question whether oxidation to thiochrome is involved in any of the metabolic functions of aneurin.

The apparent activity of the two N-substituted compounds, Nos. 4 and 5, Table III, cannot be accepted as real without examination of further purified specimens, since the method of preparation, from the highly active aneurin thiazole itself, renders contamination by the latter very probable. Furthermore similar pure quaternary salts have been found inactive in the *Phycomyces* growth test, which in general parallels the growth effects with *Staph. aureus* (E. R. Buchman, private communication).

Aneurin and its pyrimidine and thiazole components in the growth of various organisms

The observation that the pyrimidine and thiazole components of aneurin together were as effective in the growth of *Staph. aureus* as aneurin itself [Knight, 1937, 3] has been extended to other micro-organisms. It has been shown that the corresponding thiazole-pyrimidine pairs are effective in the growth of the

mould *Phycomyces blakesleeanus* [Schopfer & Jung, 1937; Schopfer, 1937; Sinclair, 1937; Robbins & Kavanagh, 1937; 1938]. Similarly Lwoff & Dusi [1937; 1938], Lwoff, A. & Lwoff, M. [1937; 1938] and Lwoff, M. [1937] have extended the field of action of aneurin or its components by showing that they are essential nutrients for certain unicellular protista [*Eucaryotes*; Lwoff, 1932].

A comparison of the need for aneurin or its components in a wide group of fungi has been made by Robbins [1938] and Robbins & Kavanagh [1938, 3], for species of the genus *Ustilago* by Schopfer & Blumer [1938], and for *Mucor Ramannianus* (Möll) by Müller & Schopfer [1937].

These investigations have shown that there are some species which need be given only one or other of the aneurin components, while other species cannot utilize the two components but must be given the complete aneurin molecule. Of the latter type are the flagellate trypanosome *Strigomonas oncopelti* [Lwoff, M. 1937]; the ciliate *Glaucoma piriformis* [Lwoff, A. & Lwoff, M. 1937, 2; 1938] and *Ustilago scabiosae* (Sow.) [Schopfer & Blumer, 1938]. Aneurin analogues inactive for *Staph. aureus* were also inactive for *Glaucoma piriformis* [Lwoff, A. & Lwoff, M. 1938].

All the strains of *Staph. aureus* examined in our laboratory (now about 30 typical strains) have required both the pyrimidine and the thiazole. In no case has a strain been observed which needed only one of them, or which could utilize only aneurin and not the two components. It is possible that other species of bacteria may be found which exhibit these growth requirements.

We are indebted to Dr G. P. Gladstone for the unpublished observations that an examination of the filtrates from cultures of *Chromobact. prodigiosum*, *Pseudomonas pyocyanea*, *B. subtilis* and *Bact. typhosum*, has shown that all these bacteria, cultivated in media which do not contain aneurin, do in fact synthesize this substance (or its two components) since the filtrates can supply the aneurin requirements of *Staph. aureus*. This is an extension of the earlier observation of Hughes [1932] with *Bact. coli*. For these bacteria therefore the dispensability of aneurin (or its components) as a nutrient essential does not mean that this substance may not be involved in their metabolism; it is in fact synthesized by these organisms.

Robbins & Kavanagh [1938, 3] found that those species of fungi which required only one or other aneurin component for growth synthesized the complementary component, since the culture filtrates could supply both components to *Phycomyces blakesleeanus*. Analogous observations for *Mucor Ramannianus* and *Rhodotorula* were made by Müller & Schopfer [1937] each organism supplying one component to the other. In general therefore it is found for the organisms discussed here that a need for one or both aneurin components, or for aneurin itself, as nutrients, reflects the relative ability of the organism to synthesize aneurin. The latter appears to be required in the metabolism of all of them, and it or its components are nutrient essentials if they cannot be synthesized.

An examination of the specificity of pyrimidines and thiazoles which can be used by various fungi and unicellular protista in place of the aneurin which provides essential nutrient material for them, and a comparison with similar observations for *Staph. aureus*, supports this view. Generally speaking the specificity in the growth of *Staph. aureus* is paralleled by the effects shown by *Phycomyces* [Robbins & Kavanagh, 1938, 1, 2; Schopfer, 1937; Sinclair, 1937] and by the protista studied by Lwoff & Dusi [1938, 2, 3]. It will therefore be simplest to note only the points of difference.

(*a*) *2-Methyl-4-amino-5-thioformamidomethylpyrimidine.* Sinclair [1937] found for *Phycomyces blakesleeanus* that this compound was less potent than the

corresponding 5-aminomethyl derivatives, in agreement with the results with *Staph. aureus*. Robbins & Kavanagh [1938, 1] confirmed this, using a sample of the 5-thioformamidomethyl derivative from the same source as the specimens used by Sinclair and by Knight, namely Dr A. R. Todd. Further, Robbins & Kavanagh showed that the activity of the 5-thioformamidomethyl derivative decreased on autoclaving to sterilize, a filtered solution being the most active. This did not occur with the 5-hydroxymethyl or 5-bromomethyl derivatives. Presumably it would not occur with the 5-aminomethyl derivative which would be the probable active contaminant of the 5-thioformamidomethyl compound if one were present (this follows from the mode of synthesis [Todd & Bergel, 1937]). Schopfer [1937] with a different specimen of the 5-thioformamidomethyl derivative (Hoffmann-La Roche) recorded only very feeble activity for *Phycomyces*, but he used an autoclaved solution. Lwoff & Dusi [1938, 2] found that a specimen of the 5-thioformamidomethyl derivative (also from Hoffmann-La Roche) but not autoclaved, had activity for *Polytomella caeca* and *Chilomonas paramoecium*, but was less potent than the corresponding 5-aminomethyl compound. With the exception of Schopfer's results, probably explicable by loss of activity on autoclaving, all the observations are in agreement that the thioformamidomethyl derivative can be utilized by *Staph. aureus*, *Phycomyces blakesleeanus*, *Polytomella caeca* and *Chilomonas paramoecium*, but in all cases is less readily available than the corresponding 5-aminomethyl, bromomethyl or hydroxymethyl derivative. This suggests a common objective in the utilization of the pyrimidine by all these organisms.

(*b*) *Thiazoles*. The most striking difference of behaviour in the utilization of thiazoles by the different organisms considered here is that shown with the compound 2:4-dimethyl-5-β-hydroxyethylthiazole. This was inactive for *Staph. aureus* (No. 6, Table III) and for *Phycomyces blakesleeanus* [Robbins & Kavanagh, 1938, 2]; but Lwoff & Dusi [1938, 3] using the same specimen as we have used in the present work find that it can be utilized by the flagellates *Polytoma caudatum*, *P. ocellatum*, and *Polytomella caeca*. The corresponding 2-hydroxy-4-methyl-5-β-acetoxyethylthiazole was inactive for these flagellates as it was for *Staph. aureus* (No. 7, Table III).

With the exception of this difference the specificity of the pyrimidine and thiazole components of aneurin is the same for all the organisms discussed here. These results emphasize the high specificity of the aneurin structure in the physiology of all these varied types of organism and also harmonize with the results obtained by Bergel & Todd [1937] with aneurin analogues for anti-neuritic activity in the rat, suggesting a fundamental metabolic role for aneurin common to all these organisms.

Compounds related to nicotinamide

The results collected in Table IV emphasize the specificity of nicotinamide and two closely related compounds in the growth of *Staph. aureus*. One of the functions of the nicotinamide must be to contribute that molecule to the synthesis of codehydrogenase I or II (pyridine nucleotide phosphate compounds) which *Staph. aureus* is known to produce. This follows from the fact that the organism is known to synthesize the "*V* factor" for *Haemophilus parainfluenzae*, which Lwoff, A. & Lwoff, M. [1937, 1] have shown to be identical with either codehydrogenase I or II. Nicotinamide is more active than nicotinic acid, in agreement with the fact that it is the amide which is required in the codehydrogenase. *Staph. aureus* can make the amide from the acid if necessary. It appears therefore that *Staph. aureus* must find nicotinic acid preformed be-

Table IV. *Pyridine derivatives*

	Conc. *M*	Growth 24 hr.
1. Nicotinamide	2×10^{-7}	+ + + +
	4×10^{-8}	+ +
	8×10^{-9}	?
2. Pyridine-3-carboxylic acid (nicotinic acid)	1×10^{-6}	+ + +
	2×10^{-7}	?
3. Methyl nicotinate	5×10^{-7}	+ (3 days)
	1×10^{-7}	? (3 days)
4. Pyridine-3-carboxylic acid diethylamide ("Coramine")	1×10^{-5}	0
5. Pyridine-3-sulphonate	1×10^{-4}	0
6. Pyridine-3-nitrile	2×10^{-5}	0
7. 3-Methylpyridine (β-picoline)	1×10^{-4}	0
8. Trigonelline methyl sulphate	1×10^{-4}	0
9. Trigonelline chloride	1×10^{-4}	0
10. Pyridine-4-carboxylic acid (*iso*nicotinic acid)	1×10^{-4}	0
11. Pyridine-2-carboxylic acid (picolinic acid)	1×10^{-4}	0
12. Pyridine-2:3-dicarboxylic acid (quinolinic acid)	1×10^{-4}	0
13. 2:4-Dimethylpyridine-3:5-dicarboxylic acid	1×10^{-4}	0
14. 2:4:6-Trimethylpyridine-3:5-dicarboxylic acid	1×10^{-4}	0
15. Nicotine	1×10^{-5}	0

None of the compounds which failed to permit growth was toxic, since growth ensued on subsequently adding nicotinamide. Failure of growth was therefore due to non-utilizability.

No. 4. "Coramine" was obtained from Messrs Ciba, Ltd., through the courtesy of Dr K. Miescher.

Nos. 6 and 9: obtained from Dr H. King.

Nos. 10 and 12: obtained from Prof. G. R. Clemo.

No. 2. A commercial specimen of nicotinic acid which even on repeated crystallization from water gave pale yellow warty clusters of needles, was obtained free from the persistently associated impurities by sublimation and recrystallization from water after digestion with norite charcoal. It then separated in long colourless flat needles, M.P. 232° (uncorr.).

No. 5. *Pyridine-3-sulphonic acid* was prepared by a method based on those given by Meyer & Ritter [1914] and Gastel & Weibaut [1934].

No. 8. *Trigonelline methyl sulphate.* Nicotinic acid (1·0 g.) and freshly distilled dimethyl sulphate (0·8 ml.) heated at 120° for 15 min., yielded a viscous gum which when crystallized from alcohol gave colourless prisms of the methosulphate, M.P. 210°. The alcoholic solution with picric acid yielded trigonelline picrate, M.P. 204°.

No. 13. 2:4-*Dimethylpyridine-3-carboxylic acid hydrochloride* was prepared according to Michael [1885].

No. 14. 2:4:6-*Trimethylpyridine-3:5-dicarboxylic acid* (*K salt*) was prepared according to Hantzsch [1882].

No. 11. The copper salt of pyridine-2-carboxylic acid was obtained from Prof. G. R. Clemo, and the free acid (M.P. 139°) was prepared from it by passing H_2S over the dry salt and extracting with water.

Nos. 7 and 15 were both commercial specimens.

cause it cannot synthesize it: the remainder of the codehydrogenase molecule (ribose, adenine) it can synthesize. The inactivity of the diethylamide ("coramine") indicates that the organism cannot remove the two ethyl groups. It can hydrolyse the methyl ester of nicotinic acid since methyl nicotinate is active, although growth is delayed. The other compounds in Table IV, all of which are inactive, emphasize how small an alteration from the structure appearing in the codehydrogenase molecules produces inactivity in growth.

Nicotinic acid has been found among the nutrient requirements of strains of *C. diphtheriae* by Mueller [1937] and of strains of dysentery bacilli by Koser *et al.* [1938]. Fildes [1937] has indicated a series of bacteria whose relative abilities to synthesize the whole or parts of the codehydrogenase I or II molecules are reflected in the nutrient requirements of the different species. It appears probable that the organisms studied by Mueller and by Koser *et al.* will be found to synthesize codehydrogenase I or II, or both.

SUMMARY

An examination of a further series of aneurin and pyridine derivatives emphasizes the previous conclusion that aneurin and nicotinamide are highly specific in the growth requirements of *Staph. aureus*. Only very limited departures from these structures are permissible if growth activity is to be maintained.

A comparison with similar observations made upon other types of organisms, in particular certain fungi and unicellular protista, suggests an underlying biochemical unity.

This work has been carried out under the direction of Dr P. Fildes who, with Dr G. P. Gladstone, has been responsible for the bacteriological side of the work.

Grateful acknowledgement is made to the numerous persons mentioned in the text who have made this work possible by supplying specimens of many of the compounds tested. We are particularly indebted to Dr E. R. Buchman and Dr A. Lwoff for close liaison in communicating to us their results in this field.

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Note added 8 June 1938. Since the above was written Woolley, Strong & Madden (*J. biol. Chem.* 1938, **123**, *Proc.* cxxxi) have reported a similar high specificity of structure for pyridine derivatives in the cure of canine black-tongue. They had previously shown that nicotinic acid or its amide could effect the cure (Elvehjem, Madden, Strong & Woolley, *J. Amer. chem. Soc.* 1937, **59**, 1767; *J. biol. Chem.* 1938, **123**, 137). The non-utilisable derivatives agree with those found for *Staph. aureus* with the exception that β-picoline has a definite effect, and nicotinic acid *N*-methylamide a slight effect with the dogs.

CLXVII. A PHYSICO-CHEMICAL AND BIOCHEMICAL STUDY OF VITAMIN A_2

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The literature concerning the existence of different substances in fish liver oils which give a colour with antimony trichloride has been reviewed recently in detail in this *Journal* by Edisbury *et al.* [1938] and by Gillam *et al.* [1938]. These investigators have established the existence in freshwater fish liver oils of a higher homologue of vitamin A, the "693 mμ chromogen" or "vitamin A_2".

Full details are given below of experiments reported briefly elsewhere [Lederer & Rathmann, 1938], which show that vitamin A_2 gives in the presence of $SbCl_3$ a second absorption band not previously observed. This band is of importance for the calculation of the relative quantities of vitamins A_1 and A_2 in any given material. Our paper deals further with data obtained from oils of freshwater fish from different parts of Europe as well as from oils or fats from other animals. Experiments dealing with the absorption of vitamin A_2 from the intestine and its accumulation in the liver of the rat and the frog are included.

Experimental

Unsaponifiable fractions were prepared by the usual methods [cf. Gillam *et al.* 1938]. The livers and intestines were hydrolysed with aqueous or alcoholic NaOH (5–10 %) and extracted with ether; the resulting concentrate was saponified. As the alkaline hydrolysis was always accompanied by a partial saponification of the oil, no values for the oil content were available. In Tables I and III are given the weights of the unsaponifiable fractions calculated for 100 g. of fresh organ. All measurements were made on the unsaponifiable fractions. For the determination of absorption coefficients a Hilger-Nutting visual spectrophotometer was used. All readings were repeated at least twice by one of us and controlled by the other. In Tables I and III it was thought sufficient to give the data for $E^{1\%}_{1\text{ cm.}}$ 695 mμ, as the corresponding values for 650 and 620 mμ can be easily calculated from the ratios E 695 mμ/E 650 mμ and E 695 mμ/E 620 mμ given in the tables.

The inhibitor (Table III, no. 18) was prepared as follows. After saponification of the pike liver oil (Table III, no. 17) the ether-extracted soap was acidified with 5 % H_2SO_4 and extracted three times with ether. The solvent was dried with Na_2SO_4 and distilled off. The colourless residue crystallized incompletely. 20 mg. dissolved in 0·1 ml. $CHCl_3$ were added to 0·2 ml. of a solution of the pike unsaponifiable no. 17 of Table III.

The 650 mμ band of vitamin A_2

There is evidence that vitamin A_2 is a higher homologue of vitamin A_1, having six conjugated double bonds, a ring of β-ionone and one hydroxyl group [Gillam *et al.* 1938]. It would be surprising if this substance had only one absorption band in presence of $SbCl_3$, seeing that vitamin A_1 and other natural

carotenoids with hydroxyl groups (zeaxanthin, lutein, violaxanthin) show two bands [Castle *et al.* 1934; Gillam, 1935; Euler & Karrer, 1932; Euler *et al.* 1932]. The hydrocarbons (α- and β-carotene, lycopene and *iso*carotene) show only one band [Karrer & Walker, 1933; Gillam, 1935]. This is also true for synthetic polyenes. Thus the tetradecapentaen-ol of Kuhn & Grundmann [1938] gives two bands with $SbCl_3$, whereas the tetradecahexaene prepared from the former by splitting off water gives only one band. The *apo*carotinols of Euler *et al.* [1938] seem to be exceptions to these rules.

We have found that vitamin A_2 has indeed a second band (situated at 645–650 mμ) but this band is usually masked, just as is the second band of vitamin A_1 at 580 mμ; it can be made visible by adding an inhibitor, in the same way as can the second band of vitamin A_1 [Emmerie *et al.* 1931; Eekelen *et al.* 1931; Castle *et al.* 1934].

Table III shows the intensity of absorption at 695 mμ of concentrates from fish liver and intestines. For those which have a relative absorption intensity E 695 mμ/E 620 mμ higher than 2, the intensity of absorption at 650 mμ is about two-thirds of that at 695 mμ, the quotient E 695 mμ/E 650 mμ varying from 1·38 to 1·80. This variation is probably not significant, so that it may be concluded that both bands are due to the same substance (see Fig. 1).

The 650 mμ band can be clearly seen on adding to the solution of the unsaponifiable in chloroform the fraction of fatty acids obtained after saponification of freshwater fish liver oils. The absorption at 695 mμ is much diminished, while absorption at 650 and at 620 mμ remains almost unchanged (Table III, nos. 17, 18).

The band at 640 or 645 mμ observed in some natural oils [Heilbron *et al.* 1931; Morton, 1932; Edisbury *et al.* 1938; Gillam *et al.* 1938] seems to correspond to the 645–650 mμ band of vitamin A_2. After saponification of these oils the inhibitor is removed and the 693 mμ band reaches its full strength and masks the second band (cf. Gillam *et al.* [1938], oil of *Huso huso*). The same band is seen in partially saponified oils together with the 693 mμ band and is then located at 645 mμ. On the other hand, the 645 mμ band is sometimes observed in partially decomposed vitamin A_2 concentrates [Gillam *et al.* 1938]; its appearance is due in these cases also to a diminution of the intensity of the 693 mμ band.

In some natural oils full inhibition of the 693 and 650 mμ bands occurs and such oils show only a weak band at 610 mμ (Table III, no. 2). After complete saponification the 610 mμ band disappears, overshadowed by the strong 693 mμ band.

Quantitative determination of vitamins A_1 and A_2

Until quite recently the determination of the relative quantities of vitamins A_1 and A_2 present in an oil was based on two assumptions: (1) that $E^{1\%}_{1\,\mathrm{cm.}}$ 693 mμ for vitamin A_2 has the same value as $E^{1\%}_{1\,\mathrm{cm.}}$ 620 mμ for vitamin A_1, i.e. about 5000 [Edisbury *et al.* 1938; Gillam *et al.* 1938]; (2) that the absorption at 620 mμ is due solely to vitamin A_1 and that at 693 mμ solely to vitamin A_2.

The first assumption seems to be fairly correct as shown by the following consideration. Vitamin A_2 having one conjugated double bond more than vitamin A_1, its molecular extinction coefficient at 345 mμ should be somewhat higher (about 20%) than that of vitamin A_1 at 328 mμ. Since, on the other hand, a 1% solution of vitamin A_2 contains about 10% fewer molecules than a 1% solution of vitamin A_1, the $E^{1\%}_{1\,\mathrm{cm.}}$ values should be about the same for both in the ultraviolet. Moreover, solutions of vitamins A_1 and A_2 having equal extinction at 328 and 345 mμ respectively show roughly the same $E^{1\%}_{1\,\mathrm{cm.}}$ at 620

and 693 mμ, as illustrated by the following figures for vitamin A_2 taken from Gillam *et al.* [1938].

Vitamin A_2	$E^{1\%}_{1\,cm.}$ 344 mμ = 850	$E^{1\%}_{1\,cm.}$ 697 mμ = 2600
Vitamin A_1	$E^{1\%}_{1\,cm.}$ 328 mμ = 850	$E^{1\%}_{1\,cm.}$ 620 mμ = 2650 (calculated on the basis of the usual E values for A_1)

The second assumption is unjustified in so far as it concerns absorption at 620 mμ, for this has been shown to be only partly due to vitamin A_1 [Lederer & Rathmann, 1938].

On measuring the absorption intensities at 620 and 580 mμ of vitamin A_1 concentrates as far as possible free from vitamin A_2 (E 695 mμ/E 620 mμ < 0·17) it was found that E 650 mμ/E 620 mμ for these preparations was approximately 0·25–0·30 (Table I, nos. 2–5). A value of 0·35 was obtained by evaluation of the absorption curve of vitamin A from frog retina measured by Wald [1936].

Table I. *Vitamin A_1 from marine fish and land animals*

No.	Species	Organ	Wt. g.	Nonsap. mg. per 100 g. organ	$E^{1\%}_{1\,cm.}$ 695 mμ	$\frac{E\ 650\,m\mu}{E\ 620\,m\mu}$	$\frac{E\ 695\,m\mu}{E\ 620\,m\mu}$
1	*Gadus merlangus*	8 livers	160	400	2·5	0·61	0·21
2	Halibut (*Hippoglossus hippoglossus*)	—*	—	—	40·0	0·29	0·17
3	*Ishinagi sterelepis*	—*	—	—	112·0	0·26	0·15
4	Sheep (*Ovis aries*)	1 liver	1080	430	1·5	0·27	0·03
5	Chick (*Gallus domesticus*)	1 liver	27	410	1·2	0·35	0·08
6	Frog (*Rana esculenta*)	14 livers	11	600	8·2	0·52	0·27

* Oil investigated.

If one supposes that the absorption curve of pure vitamin A_2 falls off at the same rate from 650 to 620 mμ as the absorption curve of vitamin A_1 from 620 to 650 mμ, one reaches the conclusion that E 620 mμ/E 650 mμ for pure vitamin A_2 must also be about 0·25–0·30.

The relative intensities of absorption of our richest vitamin A_2 concentrates were: E 695 mμ/E 650 mμ/E 620 mμ = 3/1·8/1. Now if E 620 mμ/E 650 mμ for vitamin A_2 is 0·25, then 0·45 (i.e. 1·8 × 0·25) or nearly half of the absorption at 620 mμ is due to vitamin A_2.

On the basis of these figures we suppose that E 695 mμ/E 620 mμ for pure vitamin A_2 will be between 6 and 8. (The values previously reported by Lederer & Rathmann [1938] were too low.) Taking 7 as an average value, the relative quantities of vitamins A_1 and A_2 may be calculated by the formula

$$\text{vitamin } A_2/\text{vitamin } A_1 = E\ 695\,m\mu \Big/ \left(E\ 620\,m\mu - \frac{E\ 695\,m\mu}{7}\right);$$

Table II gives the relative quantities of the two vitamins calculated directly from the ratio E 695 mμ/E 620 mμ.

Fig. 1 shows the absorption curve of a vitamin A_2 concentrate (E 695 mμ/E 620 mμ = 2·5) in presence of antimony trichloride. The crosses indicate average values from measurements on 10 different unsaponifiable fractions taken from different species of fish with ratios varying from 2·3 to 2·7. The measured curve can be shown to result from 4 separate hypothetical absorption curves with maxima at 693, 650, 620 and 580 mμ respectively (light lines). The figure demonstrates clearly the overlapping of the absorption bands of vitamins A_1 and A_2.

Table II. *Relative quantities of vitamins A_1 and A_2*

$\frac{E\,695\,m\mu}{E\,620\,m\mu}$	$\frac{\text{Vitamin } A_2}{\text{Vitamin } A_1}$	% vitamin A_2	% vitamin A_1
0·5	0·54	35	65
1·0	1·16	54	46
1·5	1·88	65	35
2·0	2·80	74	26
2·5	3·90	80	20
3·0	5·30	84	16
4·0	9·30	91	9
5·0	17·00	95	5
6·0	40·00	98	2
7·0	∞	100	0

Of course these figures have to be considered as a first approximation and will have to be revised as soon as more exact values are available. They are independent of the chemical structure of vitamin A_2, which seems to need further investigation, as pointed out by Euler *et al.* [1938].

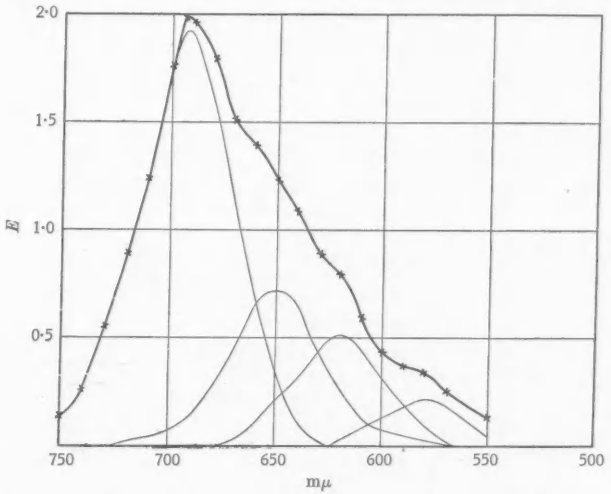

Fig. 1. Absorption spectrum of vitamin A_2 concentrates ($E\,695\,m\mu/E\,620\,m\mu = 2{\cdot}5$) in presence of $SbCl_3$ ($\times — \times — \times$). The light lines show the four hypothetical bands with maxima at 695, 650, 620, 580 mμ.

Our results are of importance in considering the biological assay of vitamin A_2 reported by Gillam *et al.* [1938]. A final conclusion as to the activity of vitamin A_2 was difficult to draw at that time as the assayed preparation was thought to contain only two parts of vitamin A_2 to one part of vitamin A_1 ($E\,695\,m\mu/E\,620\,m\mu = 2$). Since the activity of this concentrate was somewhat less than that of a halibut oil fed in equal doses, it could have been argued that the activity was due solely to the vitamin A_1 present. As the value of 2 for the ratio $E\,695\,m\mu/E\,620\,m\mu$ had been measured more than 2 weeks after the preparation of the oil, we suppose that the real value for the fresh oil was at least 2·3, as for all *Lucioperca lucioperca* concentrates measured immediately after preparation (*vide infra*). In the light of the conclusions reached above the assayed preparation contained only about 20–25 % vitamin A_1. This seems to prove that the 693 mμ chromogen deserves the name vitamin A_2 (cf. the critical remarks

of Euler *et al.* [1938]). Exact assays of our preparations have now been begun by Dr Th. Moore to determine more precisely the biological activity of vitamin A_2.

Ultraviolet absorption spectra of vitamin A_2 concentrates

Gillam *et al.* [1938] as well as Edisbury *et al.* [1938] stated that vitamin A_2 concentrates have two absorption bands in the ultraviolet, one at 345 mμ and the other at 280 mμ. The former authors are inclined to think that both bands are due to vitamin A_2 because of the more or less constant relative intensities of the two bands in concentrates from different sources, whereas Edisbury *et al.* seem to have separated the two bands by treating vitamin A_2 concentrates with 83 % alcohol. The insoluble fraction is shown to have the band at 280 mμ whereas the soluble fraction, representing vitamin A_2, has a band at 345 mμ. Edisbury *et al.* [1938] suppose that the insoluble fraction is identical with that isolated before by Pritchard *et al.* [1937] from a mammalian concentrate in the same way and that it represents a lower (C_{18}) homologue of vitamin A_1.

These conclusions seem rather improbable, because it is difficult to believe that the C_{18} homologue of vitamin A_1 should be quite insoluble in 83 % ethyl alcohol in which vitamin A_1 itself is easily soluble. Moreover, in our experiments we were unable to confirm the findings of Edisbury *et al.* concerning the separation of the two bands in the ultraviolet.

2·2 g. of a concentrate from 50 pike livers (Table III, no. 14) were dissolved in methanol and freed from sterols (1·6 g.) by freezing at $-12°$. The filtrate was then evaporated to dryness *in vacuo* and dissolved in 83 % ethyl alcohol; after standing for an hour at $-12°$ an amorphous precipitate settled out which was filtered off. The filtrate was evaporated to dryness *in vacuo* and the residue was completely soluble in 77 % ethyl alcohol at room temperature. After standing for an hour at $-12°$ a second (oily) precipitate could be separated. The filtrate contained practically the whole of the vitamin A_2 (320 mg. with $E^{1\%}_{1\,cm.}$ 695 mμ = 1060) and showed two bands in the ultraviolet (345 and 280 mμ) with relative intensities practically the same as those of the original material, as far as could be judged from the photographs taken with an Yvon and Jobin spectrograph of small dispersion.

It seems therefore that both bands in the ultraviolet belong to vitamin A_2. It may be mentioned here that Gillam [1935] found a 268–280 mμ band in the ultraviolet part of the absorption spectrum of all the carotenoids which he investigated.

The alcohol treatment resulted in a slight increase of the ratio E 695 mμ/E 620 mμ, i.e. from 2·65 for the original material (Table III, no. 14) to 3·0 for the fraction soluble in 77 % ethyl alcohol. The insoluble fraction had a ratio of 2·0.

Colour reactions of vitamins A_1 and A_2

In the hope of finding some reagent which would enable one to distinguish more specifically between the two vitamins than is possible with $SbCl_3$, a number of other substances were studied. Rosenheim & Drummond [1925] reported that trichloroacetic acid, dimethyl sulphate, perchloric acid, benzoyl chloride, acetyl chloride and arsenic trichloride gave blue solutions in the presence of vitamin A, but they recorded no spectroscopic observations, except for arsenic trichloride.

The following table gives the results of measurements made on a marine and a freshwater fish liver concentrate (E 695 mμ/E 620 mμ = 2·5 for the latter).

Reagent	Vitamin A_1	Vitamin A_2	Remarks
Trichloroacetic acid	610 mμ	690 mμ	30% reagent in $CHCl_3$
Dimethyl sulphate	615 ,,	680 ,,	20% reagent in $CHCl_3$
Perchloric acid	605 ,,	680 ,,	5–10% reagent in $CHCl_3$
Benzoyl chloride	Very weak	590 ,,	20% reagent in $CHCl_3$
Acetyl chloride	572 mμ	No band	A_1, very unstable violet colour; A_2, first blue, then violet
Arsenic trichloride	628, 577 mμ	710, then 590 mμ	A_2, first green, then violet
Reaction of Rosenthal & Erdélyi [1934]	558, 480, 450 mμ	685 mμ	A_2, no colour change on heating

These results were not encouraging for further studies, as the bands were more or less the same as those which occur in the Carr and Price reaction.

Determination of the ratio E *695 mμ*/E *620 mμ in liver oils of freshwater fish from different sources*

A striking difference seemed to exist between the results of Edisbury *et al.* [1938] on English fish and those of Gillam *et al.* [1938] on Russian fish. The former authors found in general a ratio *E* 693 mμ/*E* 620 mμ varying from 0·8 to 1·5 (for the livers of *Salmo salar*, *Salmo trutta* v. *fario*, *Salmo irideus* and *Perca fluviatilis*), whereas the livers of Russian freshwater fish (*Lucioperca lucioperca*, *Esox lucius*, *Silurus glanis*, *Abramis brama*, *Huso huso*, *Stenodus leucichthys nelma*, *Coregonus lavaretus pidschian* etc.) had ratios varying from 1·6 to 2·6. Only a few varieties of salmon and carp had low values (0·25–0·88).

We decided to investigate this point and measured the relative absorption intensities at 695 and 620 mμ of liver concentrates from a number of fish from various parts of Europe (Austria, France, Holland, Hungary and Yugoslavia). The results showed (Table III) that there was no geographical difference but a very marked species specificity as regards these relative intensities. Specimens of pike-perch (*Lucioperca lucioperca*), pike (*Esox lucius*) and catfish (*Silurus glanis*) from all over Europe had a ratio of 1·9–2·7, whereas carp (*Cyprinus carpio*), trout (*Salmo irideus*), salmon (*Salmo salar*) and sturgeon (*Acipenser sturio*) had much lower values (0·4–0·8). Marine fish from the Atlantic coast of France (*Gadus merlangus*, *Hippoglossus hippoglossus*) and from Japan (*Ishinagi sterelepis*) had still lower values (0·17–0·21). These measurements agreed well with those of Edisbury *et al.* [1938] and of Gillam *et al.* [1938] on English and Russian fish and the "striking difference" between the results of these two groups of investigators was simply due to the fact that in the two laboratories different species of fish were used.

We have observed that the beginning of oxidation in concentrates of vitamin A_2 is indicated by a decrease of the ratio *E* 695 mμ/*E* 620 mμ, due probably to the more rapid destruction of vitamin A_2. Some of the lower values for this quotient reported in the literature ([Gillam *et al.* 1938] and Table III of this paper) may be explained in this way.

Comparison of liver oils and intestinal oils

Edisbury *et al.* [1938] made the interesting observation that the intestinal oils of some fish contained great quantities of vitamin A, comparable with those found in the liver. They also recorded measurements of the ratios vitamin A_1/vitamin A_2 in the intestinal oils of freshwater fish, and found that as a rule they were lower than in the corresponding liver oils.

Our measurements confirmed the findings of Edisbury *et al.* and showed that in some species (carp, Table III, nos. 30–33) *E* 695 mμ/*E* 620 mμ of the intestinal oils was even higher than the corresponding ratio in liver oils from the same fish.

Table III. *Vitamins A_1 and A_2 in liver and intestine of freshwater fish*

No.	Name of fish	Origin	Organ	Wt. g.	Unsap. mg. per 100 g. fresh organ	$E\,\frac{1\%}{1\,cm.}$ 695 mμ	$\frac{E\,695}{E\,650}$	$\frac{E\,695}{E\,620}$	Remarks
1	Pike-perch (*Lucioperca lucioperca*)	France (Saône)	5 livers	52	340	40	1·80	2·63	Gillam *et al.* [1938] for Russian fish $\frac{E\,695\,m\mu}{E\,620\,m\mu}$ = 2·18–2·34
2	,, ,,	,,	5 livers	52	(8 g. oil)	0·5	1·00	0·58	Oil from no. 7 before saponification
3	,, ,,	,,	4 intestines	180	220	16	1·27	1·60	Whole intestine with stomach and fat
4	,, ,,	,,	14 livers	93	300	88	1·65	2·40	
5	,, ,,	,,	7 caeca	47	430	78	1·80	2·25	
6	,, ,,	,,	Exterior caecal fat from 7 caeca	110	600	5	1·18	1·00	Same fish
7	,, ,,	,,	10 stomachs	70	250	2	1·00	1·00	
8	,, ,,	Austria (Bodensee)	1 liver	27	500	110	1·60	2·60	
9	,, ,,	,,	1 intestine	56	300	115	—	2·35	
10	,, ,,	Hungary	1 liver	25	360	100	1·46	2·00	Measured 2 months after preparation of unsap.
11	,, ,,	Holland	1 liver	18	560	15	1·55	2·35	
12	Pike (*Esox lucius*)	France	7 livers	65	300	84	1·66	2·00	Measured 7 days after preparation of unsap.
13	,, ,,	,,	7 intestines	125	420	8	1·30	1·80	
14	,, ,,	,,	50 livers	440	530	180	1·56	2·65	Gillam *et al.* [1938] for Russian fish: $\frac{E\,695\,m\mu}{E\,620\,m\mu}$ = 1·90
15	,, ,,	Austria (Danube)	4 livers	40	250	112	1·38	2·00	Measured 13 days after preparation of unsap.
16	,, ,,	,,	3 intestines	120	230	24	1·48	1·88	
17	,, ,,	Holland	1 liver	12	610	185	1·54	2·65	
18	,, ,,	,,	1 liver	12	610	55	0·43	0·58	Unsap. no. 17 + inhibitor
19	Catfish (*Silurus glanis*)	Austria (Danube)	2 livers	100	750	40	1·41	2·00	Gillam *et al.* [1938] for Russian fish: $\frac{E\,695\,m\mu}{E\,620\,m\mu}$ = 2·0
20	,, ,,	,,	3 intestines	210	360	7	1·28	1·64	
21	,, ,,	Hungary	1 liver	75	670	156	1·56	2·20	
22	Perch (*Perca fluviatilis*)	Holland	5 livers	30	675	32	1·52	2·20	
23	,, (*Tinca tinca*)	France	5 livers + intestines	8	520	35	1·52	1·52	Young fish
24	Bream (*Abramis brama*)	,,	10 livers + intestines	5	460	14	—	1·40	Young fish
25	,, (*Alburnus alburnus*)	,,	80 livers + intestines	75	175	18	1·13	1·25	
26	Trout (*Salmo irideus*)	,,	10 livers + intestines	11	500	8	1·20	0·75	Young fish
27	,, ,,	,,	22 livers	31	530	9	1·05	0·54	Edisbury *et al.* [1938] for English fish: $\frac{E\,695\,m\mu}{E\,620\,m\mu}$ = 0·8
28	,, ,,	,,	25 caeca	80	370	9	0·84	0·42	
29	,, ,,	,,	25 intestines	126	220	4	0·87	0·44	Same fish
30	Carp (*Cyprinus carpio*)	,,	1 liver	30	300	7	0·67	0·42	Gillam *et al.* [1938] for Russian fish: $\frac{E\,695\,m\mu}{E\,620\,m\mu}$ = 0·25
31	,, ,,	,,	1 intestine	30	360	3	—	0·62	
32	,, ,,	Yugoslavia	2 livers	100	260	20	0·95	0·50	
33	,, ,,	,,	2 intestines	160	400	5	1·20	0·70	
34	,, ,,	Hungary	3 livers	190	380	32	1·10	0·69	
35	,, ,,	Holland	1 liver	13	685	105	0·95	0·50	
36	Sturgeon (*Acipenser sturio*)	France (Atlantic coast)	1 liver	400	260	78	1·00	0·60	Gillam *et al.* [1938] for Russian fish: $\frac{E\,695\,m\mu}{E\,620\,m\mu}$ = 1·32
37	Salmon (*Salmo salar*)	Holland	1 liver	75	210	144	1·10	0·72	Edisbury *et al.* [1938] for English fish: $\frac{E\,695\,m\mu}{E\,620\,m\mu}$ = 0·8–1·0

More detailed determinations were made on the distribution of vitamins A_1 and A_2 in the intestinal tract of the pike-perch (*Lucioperca lucioperca*). The pyloric caeca of this fish are surrounded by great quantities of fat which can be separated mechanically from the caeca themselves. For stomach and exterior caecal fat E 695 mμ/E 620 mμ was 1·0 (Table III, nos. 6, 7). The corresponding quotient for the caeca themselves and for the liver was 2·4 (Table III, nos. 4, 5).

This difference may be explained as follows. The vitamin A of the food is composed of about equal parts of vitamins A_1 and A_2 (E 695 mμ/E 620 mμ = 1 for the stomach contents). After absorption, a part of the fat of the food is deposited in the fat depots around the caeca, a process which leaves the ratio vitamin A_1/vitamin A_2 unchanged. In the liver and the caeca, on the other hand, there seems to be a constant coming and going of fat resulting in a greater accumulation of vitamin A_2 owing to its more unsaturated character. This would be in agreement with the observations of Zechmeister & Tuszon [1935, 1] that the more unsaturated carotenoids lycopene and capsanthine are more easily stored in human fat than carotene or lutein, as well as with the experiments of Thorbjarnarson & Drummond [1938] which show that fat leaving the liver may carry away some vitamin A.

In one experiment the caeca of a pike-perch were emptied of their contents and the caecal skin and the contents were analysed separately, with the following results:

	Wt. g.	Nonsap. mg.	$E^{1\%}_{1\,cm.}$ 695 mμ	E 695 mμ/ E 620 mμ	Mg. vit. A_2 per 100 g.*
Caecal skin	2·1	27†	50·5	2·4	12·5
Caecal contents	1·9	12†	24·0	2·1	3·4

* Calculated on the assumption that $E^{1\%}_{1\,cm.}$ 695 mμ of pure vitamin A_2 is 5000.
† Not entirely saponified.

The pyloric caecal skin had a high absolute content of vitamin A_2 which was more than twice as great as that of the corresponding liver (Table III, no. 4; 5·3 mg. vitamin A_2 per 100 g. fresh organ). It is reasonable to suppose that the accumulation of this vitamin in the caeca is not fortuitous but is related to the mechanism of fat absorption discussed by Edisbury *et al.* [1938] in connexion with vitamin A_1.

E *695 mμ*/E *620 mμ of liver fat of animals other than fish*

The liver fat of sheep and chicken contains no vitamin A_2 (Table I, nos. 4, 5), that of the green frog only small quantities (Table I, no. 6).

These observations confirm and extend the findings of Castle *et al.* [1934], Edisbury *et al.* [1938] and Gillam *et al.* [1938] concerning the absence of the 693 mμ chromogen from mammalian liver fat and seem to indicate that vitamin A_2 is a specific product of the liver metabolism of certain species of freshwater fish.

Exact measurements of the vitamin content of invertebrates have often been impossible because of the minute quantities of chromogenic material isolated or because of the presence of substances causing the formation of a turbidity with $SbCl_3$. Qualitative observations indicate that the intestines of the cephalopod *Loligo vulgaris* contain vitamin A_1 without any trace of vitamin A_2.

Accumulation of vitamin A_2 in the liver of the rat and the frog

The absence of vitamin A_2 from mammalian liver fat might be due to a differential absorption of vitamins A_1 and A_2 from the intestine (cf. the differential absorption of carotene and xanthophyll by the horse intestine observed by

Zechmeister & Tuszon [1935, 2]) or to a more rapid destruction of vitamin A_2 after absorption.

Qualitative feeding experiments have shown that the rat accumulates vitamin A_2 in its liver when receiving orally a concentrate from freshwater fish liver oil in the same way that it accumulates vitamin A_1. The absence of vitamin A_2 from mammalian liver fat can therefore be explained by the absence of vitamin A_2 from the food.

Feeding experiments on frogs, using concentrates of both vitamin A_1 and vitamin A_2, have shown that the amount of vitamin A_1 as well as the ratio vitamin A_2/vitamin A_1 can be increased in the liver.

Increase of the ratio E *695 mμ*/E *620 mμ in the liver fat of frogs fed on a vitamin A_2 concentrate*

No. of frogs	No. of feedings*	*E* 695 mμ/*E* 620 mμ
4	0	0·24
2	4	0·30
2	8	0·30
4	18	0·55

* Two drops of pike-perch liver oil (Table III, no. 2) a day per frog; no other food.

Owing to the slow metabolism of these animals little effect was produced till the feedings were continued for more than a week. Most of the oil fed was found in the intestine of the frogs.

Summary

1. Vitamin A_2 (693 mμ chromogen) gives with antimony trichloride a two-banded spectrum (maxima at 693 and 650 mμ). In unsaponifiable fractions the band at 650 mμ is entirely masked by the 693 mμ band; it can be seen in some natural oils and in partly saponified oils owing to the presence of inhibitors which diminish the intensity of the 693 mμ band. It can be made visible in unsaponifiable fractions by the addition of such inhibitors.

2. The 650 mμ band of vitamin A_2 causes considerable absorption at 620 mμ and therefore only a part of the absorption at 620 mμ observed in vitamin A_2 concentrates is due to the presence of vitamin A_1. Measurements and calculations lead to the conclusion that in concentrates rich in vitamin A_2 (*E* 695 mμ/*E* 620 mμ = 3) nearly half of the absorption at 620 mμ is due to vitamin A_2. Thus *E* 695 mμ/*E* 620 mμ of pure vitamin A_2 would lie between 6 and 8.

3. The ultraviolet absorption spectrum of our purest vitamin A_2 concentrates, which are entirely soluble in 77 % ethyl alcohol, shows absorption bands at 345 and 280 mμ.

4. Trichloroacetic acid, dimethyl sulphate, perchloric acid and other reagents give with both the vitamins coloured solutions having about the same bands as those which occur in the Carr and Price reaction.

5. The relative quantities of vitamins A_1 and A_2 (expressed as *E* 695 mμ/*E* 620 mμ) have been measured for a series of livers from freshwater fish from several countries of Europe. It has been found that this ratio is more or less constant for a given species. Thus pike-perch (*Lucioperca lucioperca*), pike (*Esox lucius*), catfish (*Silurus glanis*), and perch (*Perca fluviatilis*) have ratios of 2·0–2·7; trout (*Salmo irideus*), carp (*Cyprinus carpio*), sturgeon (*Acipenser sturio*) and salmon (*Salmo salar*) have low ratios (0·4–0·8). Marine fish (*Gadus merlangus, Hippoglossus hippoglossus, Ishinagi sterelepis*) and land animals such as sheep

(*Ovis aries*), chicken (*Gallus domesticus*) and frog (*Rana esculenta*) have still lower ratios (0·03–0·27).

6. Intestinal oils of freshwater fish have usually a somewhat lower ratio $E\,695\,m\mu/E\,620\,m\mu$ than the liver oils of the same animals. For the carp, on the contrary, this ratio tends to be higher in the intestine than in the liver.

7. The stomach contents and depot fat around the pyloric caeca of the pike-perch have a ratio $E\,695\,m\mu/E\,620\,m\mu = 1{\cdot}0$, whereas the liver and caeca themselves have a ratio of 2·4. A possible explanation for this difference is discussed.

8. Feeding experiments have shown that the rat and the frog can absorb vitamin A_2 from the intestine and accumulate it in the liver. Thus the absence of vitamin A_2 from the liver of mammals and other land animals can be explained simply by the absence of vitamin A_2 from the food.

Vitamin A_2 seems to be a specific product of the liver metabolism of certain species of freshwater fish.

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CLXVIII. THE UTILIZATION OF CO_2 BY THE PROPIONIC ACID BACTERIA[1]

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The utilization of CO_2 by heterotrophic bacteria was first reported by Wood & Werkman [1936, 1] in the case of the fermentation of glycerol by the propionic acid bacteria. Barker [1936] and van Niel [1937] have questioned the evidence for this physiologically important phenomenon. Van Niel states, "Wood and Werkman claim that carbon dioxide is reduced during the fermentation of glycerol by propionic acid bacteria. The published results cannot, however, be considered conclusive, although the data do seem to favor their claim." According to previous concepts of the physiology of heterotrophic, non-photosynthetic bacteria the utilization of CO_2 by the propionic acid bacteria appears unlikely; thus confirmation by other workers is desirable. Neither Barker nor van Niel state the respect in which they consider our data inconclusive. It should be clearly recognized that although theories advanced concerning the mechanism of CO_2 assimilation are necessarily speculative and analyses of the fermentations will show experimental variation, proof of CO_2 utilization does not depend on these points. The proof is simply that a given quantity of CO_2 in the form of $CaCO_3$ was added to a medium which was fermented and analysed. Less CO_2 was accounted for than was originally present in the medium. Moreover, the recovery of the CO_2 from the $CaCO_3$ in an unfermented control medium was quantitative. The fact that the C recovery and oxidation-reduction balance were satisfactory when CO_2 utilization was assumed but were not when the principle of CO_2 utilization was not employed, was considered further evidence substantiating the occurrence of the phenomenon.

The purpose of the present investigation was to obtain additional information concerning the utilization of CO_2 in the dissimilation of glycerol by propionic acid bacteria. It is hoped that these studies will ultimately lead to an elucidation of the mechanism of this utilization.

Methods

Two procedures have been used involving (1) serial analysis of the fermentation and (2) single analysis at the conclusion of the fermentation. The medium consisted of glycerol 2·5%, Difco yeast extract 0·4% and $NaHCO_3$ 1·5%. Each constituent was sterilized separately. The $NaHCO_3$ solution was made acid to phenolphthalein by saturation with CO_2 before adding it to the medium. Incubation was at 30°. The inocula were suspensions of cells in distilled water, obtained from glycerol yeast extract medium, used in a concentration of 3% by volume. During incubation the medium was agitated by shaking once each day.

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The apparatus used for the serial analysis is shown in Fig. 1. The fermentation was conducted under an atmosphere of CO_2 in a 2 l. Erlenmeyer flask (*A*) with 1900 ml. medium. The rubber stopper held an inlet (*B*) for CO_2-saturation of the medium, an outlet (*C*) leading to the absorption train (*D*), a syphon (*E*) for sampling, one connexion leading to a Hempel gas burette (*F*) and reservoir (*G*),

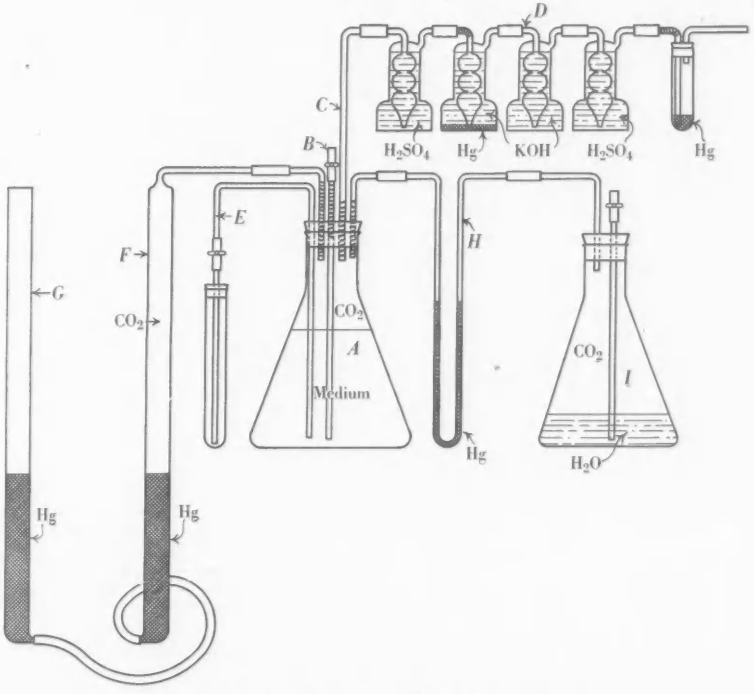

Fig. 1. Fermentation apparatus used in serial experiments.

a second connexion to a U-tube manometer (*H*) which was in turn connected to a closed flask (*I*) containing CO_2 and water to keep the gas saturated. The pressure in the fermentation flask was balanced against that of reference flask *I* by adjustment of the reservoir (*G*) before reading the volume in the burette and removing a sample. It is convenient to use a reference flask since the apparatus is then not affected by changes in temperature or pressure. There was a liberation of CO_2 during most of the fermentation, in spite of its utilization, owing to the reaction of the $NaHCO_3$ with acids formed by fermentation. This CO_2 was collected in 30% KOH in two Bowen bulbs in the drying train (*D*) and determined by weight. Hg was placed in the bottom of the first KOH bulb to cover the tip of the inlet tube to act as a trap between the alkali and CO_2 and prevent the alkali from being pulled back into the gas train. Only after the $NaHCO_3$ had completely reacted was the utilization of gaseous CO_2 apparent. At this time a partial vacuum was created in the fermentation flask and the gas utilized was measured in the burette.

All samples were removed into a known volume of alkali to prevent loss of dissolved CO_2. During removal of the sample, CO_2 was simultaneously introduced into the flask, thus preventing change in the equilibrium of free and dissolved gas. The liquor was thoroughly agitated before sampling to facilitate gaseous equilibrium. The original glycerol and CO_2 in the medium, i.e. dissolved CO_2 plus CO_2 bound as $NaHCO_3$ were determined on a sample removed immediately after the medium was inoculated and saturated with CO_2.

The principles of the apparatus are described in more detail by Dixon [1934] and Wood *et al.* [1936]. No rubber tubing was used except at glass to glass joints where flexibility was necessary. Burette and manometer were filled with Hg.

In the second procedure when only a single analysis of the fermented medium was made, the apparatus shown in Fig. 2 was used. All connexions were sealed by Hg. The fermentation flask was a 300 ml. Erlenmeyer with a tube fitted by a

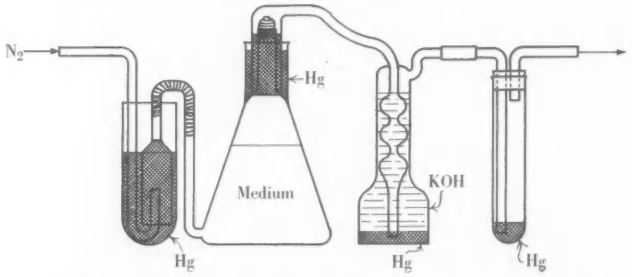

Fig. 2. Fermentation apparatus used in single analysis experiments.

ring seal into the neck. 250 ml. of medium were used in each flask. The incubation time was 30 days. Anaerobiosis was assured by passing O_2- and CO_2-free N_2 through the medium before the addition of the $NaHCO_3$. After this addition the medium was again flushed with N_2 for about 5 min.; longer treatment caused the medium to become alkaline owing to the removal of CO_2. The original CO_2 and glycerol in the $NaHCO_3$ and glycerol solutions were determined before addition to the medium. During the fermentation a slight vacuum was maintained in the apparatus (Fig. 2) thus preventing the development of pressure sufficient to break the Hg seals. After completion of fermentation, the CO_2 was flushed from the medium for 3 hr. with N_2. Because of difficulty of including a drying train in the apparatus (Fig. 2), the CO_2 collected in the KOH was liberated and reabsorbed in a suitable train for determination by wt., adding excess H_2SO_4 (blue to Congo red), boiling the whole under a reflux condenser and aerating. A correction was made for the small amount of CO_2 contained originally in the 30% KOH.

Other quantitative determinations in both procedures were made as follows. The CO_2 (dissolved CO_2 and $NaHCO_3$) of the fermented medium was determined by wt. on an aliquot part of the sample. The volatile acids and propyl alcohol were determined on the same fraction after removal of the CO_2. The alcohol was concentrated and separated from the volatile acids by distillation of one-half of the volume of the liquor and redistillation after neutralization. The alcohol was determined by oxidation with dichromate and analysis of the resulting acids (unpublished method). The volatile acids were obtained from the acidified

combined residues of the two alcohol distillations by steam distillation and were determined by the partition method of Osburn *et al.* [1933].

Succinic acid was extracted with ether from an aliquot part of the sample acidified with H_2SO_4 and determined gravimetrically as the Ag salt after removal of the volatile acids from the extract by steam distillation. The titratable acid in the distilled extract was equivalent to the succinic acid obtained in the Ag salt, also the Ag content of the salt was in agreement with the theoretical value. A separate aliquot part of the unheated liquor was extracted because apparently the glycerol and succinic acid react during the distillation. Previous results reported by Wood & Werkman [1936, 1] may show some error in the succinic acid since the determinations were made on extracts obtained from the residues of steam distillations which were taken up in Na_2SO_4. Recovery of succinic acid by this method is low when glycerol is present but complete when absent.

The unfermented glycerol was determined in an aliquot part of the medium by oxidation with periodic acid (unpublished method). This oxidation is specific for polyhydroxy and related compounds. The medium in the absence of glycerol gives a negligible determination and none of the products reacts. Pyruvic and lactic acids were not found at any time during the fermentation and there was no reduction of Fehling's solution by the fermentation liquor after hydrolysis with acid. Total C determinations were made by the method of Osburn & Werkman [1932] on the acidified medium from which residual CO_2 was removed. All analyses were made on the unfermented medium and the small blank values thus obtained were subtracted from the determinations made on the fermented medium.

Experimental results

Results of serial analysis of the fermentation by *Propionibacterium pentosaceum* are presented in Tables I and II. The data in Table I are calculated on the basis of 1 l. medium for the period t_0 to the time of removal of the respective samples. The results in Table II are calculated on the basis of the fermentation occurring during the time between samplings and per 100 m*M*. glycerol fermented. Data obtained by single analyses of the fermentation are given in Table III.

The accuracy of the data can be judged from the C recovery and oxidation-reduction balance; the former (Table I) is low during the early period but increases as the fermentation proceeds. On the basis of the glycerol fermented and CO_2 utilized the C unaccounted for is substantially constant after the 9th day as shown in Table I. The maximum cell development is reached on the 7th or 8th day and remains quite constant thereafter. This simultaneous occurrence

Table I. *Serial analysis of the dissimilation of glycerol by* Propionibacterium pentosaceum (*49 W*)

Time days	Glycerol fermented per l. m*M*.	CO_2 utilized per l. m*M*.	Products per l. Succinic acid m*M*.	Propionic acid m*M*.	Acetic acid m*M*.	Propyl alcohol m*M*.	C unaccounted for per l. m*M*.	C recovery %	Redox-index O/R
4	36·55	5·34	4·98	24·9	1·49	1·42	13·0	88·7	1·04
6	66·26	10·53	10·20	43·8	2·46	3·84	20·5	90·2	1·01
9	114·40	23·90	23·10	73·8	3·56	6·61	26·4	92·8	0·97
12·5	160·90	41·80	41·00	101·9	4·31	6·64	26·2	95·0	0·98
19·5	191·60	53·60	51·60	120·1	4·16	7·66	30·1	95·2	0·97
28·5	222·30	70·50	68·60	136·7	8·18	4·66	22·1	97·0	1·00
46·5	275·20	103·80	99·80	159·0	5·94	4·69	26·9	97·1	0·99

Table II. *Serial analysis of the dissimilation of glycerol by* Propionibacterium pentosaceum (*49 W*)

Interval days	Glycerol fermented per l. m*M*.	CO_2 utilized per 100 m*M*. glycerol m*M*.	Products per 100 m*M*. glycerol: Succinic acid m*M*.	Propionic acid m*M*.	Acetic acid m*M*.	Propyl alcohol m*M*.	C recovery %	Redox-index O/R
0–4	36·55	14·6	13·6	68·3	4·08	3·87	88·7	1·04
4–6	29·71	17·5	17·6	63·6	3·26	8·15	92·0	0·96
6–9	48·10	27·8	26·7	62·2	2·29	5·76	96·2	0·94
9–12·5	46·50	38·3	38·6	60·5	1·61	0·06	100·2	1·01
12·5–19·5	30·60	38·5	34·7	59·4	−0·49	3·34	96·3	0·92
19·5–28·5	30·70	55·5	55·2	54·1	13·09	−9·77	106·4	1·12
28·5–46·5	52·90	62·9	59·0	42·1	−4·23	0·06	97·7	0·95

Table III. *Dissimilation of glycerol by the propionic acid bacteria.**

Culture*	Glycerol fermented per l. m*M*.	CO_2 utilized per 100 m*M*. glycerol m*M*.	Products per 100 m*M*. glycerol: Succinic acid m*M*.	Propionic acid m*M*.	Acetic acid m*M*.	Propyl alcohol m*M*.	C recovery %	Redox-index O/R
11 W	214·3	12·6	16·6	80·00	3·97	1·42	101·9	1·06
34 W	212·5	10·4	15·0	76·40	3·30	3·32	98·4	1·08
35 W	212·3	13·5	16·9	77·10	2·74	2·77	99·7	1·04
49 W	211·5	17·2	20·2	76·60	1·38	1·95	100·7	1·03
52 W	206·7	21·1	25·1	74·19	2·08	0·19	102·0	1·07

* Culture numbers correspond to the following species: 11 W, *P. peterssonii*; 34 W, *P. arabinosum*; 35 W, *P. zeae*; 49 W, *P. pentosaceum* and 52 W, *P. shermanii*.

of cell development and low C recovery suggests that for the most part the C unaccounted for occurs as cell substance. The redox-index is satisfactory in almost every case in Table I. There is more variation in the balances shown in Table II than in those of Table I which is the result of the small values involved in the calculations of the data of Table II. The largest discrepancy is in the period between 19·5 and 28·5 days. When the data are considered as a whole and the C in cell material is taken into account the results are acceptable. The redox-indexes in Table III show some variations from 1·0; however, a small error in the analysis can cause such discrepancies.

A large utilization of CO_2, which started with initial fermentation and increased thereafter, is shown by Tables I and II. Since Barker and van Niel have previously questioned the validity of similar evidence of CO_2 utilization it seemed advisable to obtain further proof. The organic C in the medium should increase by an amount equivalent to the CO_2 utilized, since there is no loss of C as gaseous products. This relationship has been established by total C determinations on the medium before and after fermentation. The C determinations were made on the medium after bound CO_2 ($NaHCO_3$ and dissolved CO_2) had been removed. Aliquot parts of the original sample (unfermented) and the final sample (fermented for 46·5 days) equivalent to exactly 3 ml. undiluted medium were used in the determinations. The $K_2S_2O_8$ oxidation gave 0·1235 g. CO_2 per 3 ml. in the unfermented medium and 0·1355 g. CO_2 in the fermented medium, an increase of 0·0120 g. Experimentally 103·8 m*M*. of CO_2 per l. were utilized (Table I, 46·5 days) or 0·0137 g. CO_2 per 3 ml. The fact that the CO_2 utilized, as determined experimentally, agrees closely with the increase in organic C, substantiates the utilization of CO_2 and is strong evidence for its conversion into organic compounds.

Further evidence of CO_2 utilization was shown by the formation of a partial vacuum in the fermentation flask in the interval between 12·5 and 19·5 days. During this time the $NaHCO_3$ in the medium was entirely decomposed and the utilization was no longer masked by evolution of CO_2; 123 ml. CO_2 at N.T.P. were utilized per l. medium.[1]

Propyl alcohol was a product of the fermentation of glycerol in the present investigation. The authors have not found previous mention of the formation of this compound by propionic acid bacteria. Its isolation and identification will be described elsewhere. The alcohol obtained in the present investigation yielded after oxidation with dichromate a mixture of propionic and acetic acid in exactly the same proportion as from authentic propyl alcohol.

Discussion

The experimental results show clearly that substantial quantities of CO_2 are utilized by the propionic acid bacteria in the dissimilation of glycerol. It is of primary interest to obtain an insight into the mechanism of CO_2 utilization and to determine whether the phenomenon occurs in other biological processes.

The results in Tables I, II and III bring out one point which may give an indication of the mechanism of CO_2 utilization. The m*M*. of CO_2 utilized and succinic acid formed show a close relationship in all the fermentations. This approximate equivalence of the succinic acid and CO_2 suggests that the former may originate by union of CO_2 with some 3-C compound formed from the glycerol. In this connexion the possibility must be considered of the formation of CO_2 from glycerol and its utilization together with that from the $NaHCO_3$. Since a 2-C compound (acetic acid) is formed from a 3-C substrate (glycerol), it is only reasonable to expect the formation of an equivalent concentration of a 1-C compound (CO_2). In this case the total CO_2 utilized will be equal to the sum of the CO_2 obtained from the $NaHCO_3$ (3rd column, Tables I-III) and that originating from glycerol with the acetic acid. Comparison of this sum (Table III) with the values obtained for succinic acid (16·6–16·6, 13·7–15·0, 16·3–16·9, 18·6–20·2, 23·2–25·1) discloses that the agreement is within limits of experimental error. The results shown in Table I[2] are not in complete agreement with this explanation: If to the CO_2 utilized from the $NaHCO_3$ a quantity is added equivalent to the observed acetic acid, then there is obtained a significant excess of total CO_2 over the succinic acid. It is probable that the results in Table III are more reliable than those in Table I inasmuch as the latter were obtained by a more complex procedure. However, the redox-indexes are more satisfactory in Table I. It is not deemed advisable to propose reactions to explain these small variations until additional evidence is available. Possibly part of the succinic acid was broken down to acetic acid without evolution of CO_2. There is, however, a general correlation of CO_2 utilization with succinic acid formation suggesting a synthesis involving CO_2 and succinic acid. Results reported in a previous paper [Wood & Werkman, 1936, 1] do not show an equimolar formation of CO_2 and succinic acid. However, as previously indicated, the method used in the determination of succinic acid was not accurate in all cases. For this reason present experiments are a better criterion of the CO_2-succinic acid relationship.

[1] The *p*H of the medium was 5·5 on the 19·5 day. Sufficient alkali was therefore added to buffer the medium throughout the remainder of the fermentation and it was again saturated with CO_2. A sample was then removed for determination of bound CO_2.

[2] Table II is not considered here because the method of calculation multiplies the experimental error.

During the preparation of this manuscript the publication of Elsden [1938] appeared, showing that the rate of succinic acid formation by washed suspensions of *Bacterium coli commune* from sodium pyruvate, glucose and galactose is a function of the concentration of CO_2 in the medium. No direct evidence was obtained for fixation of CO_2. It was suggested that possibly CO_2 is involved in the synthesis of succinic acid but the facts were too few to permit theorizing on the role of CO_2 in succinic acid formation. In the view of present results which show that succinic acid, a 4-C compound, is formed from the 3-C compound glycerol in amounts approximately equivalent to the CO_2 utilized, the definite possibility that succinic acid may be formed by synthesis from 3-C and 1-C compounds is evident. In the light of this suggestion an explanation is available for the results obtained by Virtanen [1925] and Virtanen & Karström [1931] who found succinic and acetic acids in equimolar quantities as the only products from glucose using preparations of propionic acid bacteria in the presence of toluene. These results could not be explained logically on the basis of a 3-C cleavage, i.e.

one 6-C = two 3-C = two 2-C + two 1-C; and two 2-C = one 4-C,

since CO_2 utilization was not considered at that time. The formation of succinic and acetic acids by a 4- and 2-C cleavage was therefore proposed to account for the formation of succinic and acetic acids unaccompanied by a 1-C compound. It is quite evident that Virtanen & Karström's results are precisely those to be expected in view of the present proposal, i.e. that one hexose yields two 3-C compounds; one is converted to acetic acid and CO_2 and the other unites with CO_2 or some derivative of CO_2 to give succinic acid.[1] Other investigators, Virtanen [1928], Scheffer [1928], Braak [1928] and Kluyver [1931], have applied the idea of 4- and 2-C cleavage to bacterial fermentations primarily because it offered an explanation for the shortage of 1-C compounds.

It is of interest that Butkewitsch & Gaewskaya [1935] and Wells *et al.* [1936] claim that the formation of citric acid from sugar by moulds cannot proceed by synthesis from 2-C compounds which originate from 3-C compounds. This view is maintained since they obtained more than the theoretical yield of citric acid, i.e. if 1-C compounds are not utilized. Gudlet & Makarowa [1935] proposed a 4-and 2-C cleavage of glucose with a subsequent synthesis as a possible mechanism in the formation of citric acid. Previously Bernhauer & Slanina [1934] showed that fungi can synthesize oxalic acid from formic acid and pointed out that there is no arbitrary limit to the quantity of citric acid which may be formed from glucose if synthesis occurs. The merits of the different schemes of citric acid formation are not within the scope of this investigation but it should be emphasized that it is not possible to limit the quantity of citric acid which can be formed from glucose since CO_2 utilization may also occur in mould metabolism. Woods [1936] has shown that *Escherichia coli* can reduce CO_2 to formic acid. This conversion does not involve a C to C linkage but does show that CO_2 can be activated by *Esch. coli*. Wieringa [1936] claims to have isolated an anaerobic spore-forming bacterium which almost quantitatively forms acetic acid from CO_2 and H_2. The methods and data necessary to support the claim are not presented in his brief report but his discovery may be another example of the chemosynthetic activity of heterotrophic bacteria. If the propionic acid bacteria and the anaerobic sporulating bacterium of Wieringa are oligocarbophilous there

[1] There is one inconsistency in Virtanen & Karström's results. They obtained an oxidized product, succinic acid, with no corresponding reduced product, acetic acid being neither oxidized nor reduced.

is no reason *a priori* to assume that moulds and other tissues are not similar. There is no direct proof of the occurrence of a 4- and 2-C cleavage in biological reactions and many of the past concepts of fungous and bacterial metabolism may have to be re-evaluated on the basis of CO_2 utilization. Krebs & Johnson [1937] have recently shown that citric acid is synthesized by avian tissue from oxaloacetic acid and some unknown compound. It is possible that this synthesis involves utilization of CO_2.

Previously Wood & Werkman [1936, 1, 2] and Wood *et al.* [1937] have shown that certain glucose fermentations by propionic acid bacteria yield relatively large quantities of succinic acid and CO_2 and a small quantity of acetic acid. These results are readily adaptable to a scheme of fermentation involving the formation of succinic acid from acetic acid. On the contrary, if the succinic acid is assumed to be formed by union of CO_2 (or some other 1-C compound) and a 3-C compound, it is difficult to explain the origin of the large quantities of CO_2 in comparison with the small amount of acetic acid. Two mechanisms have previously been proposed for the formation of CO_2; one by the breakdown of pyruvic acid to acetic acid and CO_2; the other by decarboxylation of succinic acid to propionic acid and CO_2. If these reactions were the only sources of CO_2, and succinic acid was formed by union of CO_2 and a 3-C compound, the CO_2 would of necessity be less than the acetic acid provided that the latter was not broken down. If succinic acid formation occurs in glucose dissimilation solely by CO_2 synthesis it is probable that there is a mechanism of CO_2 formation which has not been considered as yet. Stone *et al.* [1936] and Wood *et al.* [1937] have shown that acetic acid is activated and dissimilated by propionic acid bacteria. In fact the results in Table I indicate there may have been some breakdown of acetic acid between 28·5 and 46·5 days. Aerobically, resting cells of propionic acid bacteria can attack acetic acid slowly (unpublished results). At present it seems probable that there may be two mechanisms of succinic acid formation in glucose dissimilation: one by CO_2 synthesis and another by synthesis involving acetic acid [Wood & Werkman, 1936, 2].

Although the present observations showing the equivalence of CO_2 and succinic acid indicate that the latter is formed predominantly by CO_2 synthesis in glycerol fermentation, other interpretations can be given. The formation of the oxidized product, succinic acid, must be accompanied by a simultaneous reduction. Since the utilization of CO_2 involves reduction, the relationship of CO_2 and succinic acid may arise in the establishment of the oxido-reduction balance.

The close connexion of CO_2 to succinic acid formation has also been shown by fermentation of glycerol under N_2 with phosphate as a buffer. Numerous experiments have been conducted but in no case have substantial quantities of succinic acid been found as a product under these conditions, i.e. in the absence of CO_2. Propionic, acetic, pyruvic and lactic acids and propyl alcohol are formed with no gas. These experiments indicate that CO_2 is necessary for the formation of succinic acid and are in complete agreement with the investigations of Elsden [1938].

The relative change in the course of the fermentation with time as shown in Table II is of interest. On the basis of the glycerol fermented, the CO_2 utilized and succinic acid formed during each interval progressively increased whereas the propionic acid decreased. Comparing the first with the final analysis, the mM. CO_2 utilized increased from 14·6 to 62·9 and the succinic acid from 13·6 to 59·2 mM. while the propionic acid decreased from 68·3 to 42·1, per 100 mM. glycerol. The cause of this change is not clear but it illustrates how large the utilization of CO_2 can be and also the relative changes in the other products.

The CO_2 amounted to 17·3 % of the total C converted, on the basis of the glycerol fermented and CO_2 utilized between 28·5 and 46·5 days.

The complete mechanism of the fermentation of glycerol by propionic acid bacteria is not clear. The role of phosphate esters in this fermentation has not been investigated extensively. Werkman *et al.* [1937] have shown that the fermentations of glycerophosphoric and phosphoglyceric acids are inhibited by 0·02 *M* NaF. On the contrary, the fermentation of glycerol with proliferating cells proceeds in this concentration of fluoride (unpublished results). Apparently glycerophosphoric acid and phosphoglyceric acid are not necessary intermediary compounds. Wiggert & Werkman [1938] have recently shown that propionic acid bacteria grown in the presence of fluoride do not have an enzyme system for the breakdown of phosphoglyceric acid, whereas in the absence of fluoride this enzyme system develops. Perhaps this also occurs in the fermentation of glycerol, in which case, in the absence of fluoride, phosphoglyceric acid and glycerophosphoric acid may be intermediates. Wood & Werkman [1938] have shown that glycerophosphoric acid is fermented to the same final products as glycerol and, on this basis, they could not exclude the ester as an intermediate of glycerol fermentation. Whether or not CO_2 uptake is connected with phosphorylation is not known at present. Pyruvic acid probably occurs as an intermediate since it may be isolated from glycerol fermentations by sulphite fixation [Wood *et al.* 1937]. This compound may be the point of entrance for CO_2. Apparently propyl alcohol can be dissimilated by propionic acid bacteria. There was a decrease in the actual amount of propyl alcohol at the conclusion of the fermentation (Table I). Propyl alcohol is probably formed by reduction of propaldehyde which has been isolated from glycerol fermentation by fixation with sulphite and dimedon [Wood & Werkman, 1934].

Recently Fromageot & Bost [1938] have reported that the succinic acid which they obtained in propionic fermentation probably originated from compounds of the yeast extract. The CO_2 was not determined in their experiments but was calculated from arbitrary equations. Wood & Werkman [1936, 2] have shown that similar equations are not completely adaptable to the propionic acid fermentation of glucose. The excessive C recoveries (based on calculated CO_2 values) obtained by Fromageot & Bost are not a strictly reliable indication that products are being formed from the yeast extract, inasmuch as no consideration was given to the possibility of CO_2 utilization. It is probable that small quantities of different products are formed from the yeast extract; however, there is no evidence that these products are confined to succinic acid. In our experiments in which Difco yeast extract (0·4 %) has been used as the source of N there has been no evidence that the quantities of products formed from the yeast extract are significant when compared with a fermentation of 2·5 % substrate.

Summary

Previous experiments showing utilization of CO_2 by propionic acid bacteria fermenting glycerol have been confirmed and extended. The C of the utilized CO_2 has been found in the products, which are succinic, propionic and acetic acids and propyl alcohol.

Total C determinations before and after fermentation show that there is an increase in the total organic C of the medium equivalent to the decrease in inorganic C (CO_2).

The uptake of gaseous CO_2 was sufficient to create a partial vacuum in the apparatus.

The succinic acid formed and CO_2 utilized are approximately equimolar. It is suggested that the formation of succinic acid is by synthesis from a 3-C compound through addition of CO_2.

In the absence of CO_2 little or no succinic acid is formed.

The relative proportions of the products varied with time and the CO_2 utilized. The succinic acid formed increased while the propionic acid decreased.

Utilization of CO_2 by heterotrophic bacteria is considered in connexion with other reactions proposed for the formation of succinic and citric acids.

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